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CONTENTS OF VOL. 105

No. 1. 15 *July* 1946

PAGE

The partition of potassium between the isolated lung of the dog and its perfusate with particular reference to the action of adrenaline. By **Catherine Hebb** and **R. H. Nimmo-Smith** 1

The effect of dietary restriction upon the incisor teeth of rachitic rats. By **J. T. Irving** 16

Rod and cone components in the electric response of the eye. By **E. D. Adrian** 24

The presence of a sympathomimetic substance in extracts of mammalian heart. By **U. S. v. Euler** 38

The distribution of excitation and inhibition in single-fibre responses from a polarized retina. By **Ragnar Granit** 44

An investigation for the presence of a skin protease inhibitory factor in burned skin. By **A. Beloff** and **R. A. Peters** 54

Changes in intestinal tone and motility associated with nausea and vomiting. By **R. A. Gregory** 58

On the occurrence in vitro of cells resembling osteoclasts. By **N. M. Hancox** 66

Observations on plasma volume estimations with the dye T 1824. By **J. Armin** and **E. B. Reeve** 72

Reflexes elicited by visceral stimulation in the acute spinal animal. By **C. B. B. Downman** and **B. A. McSwiney** 80

No. 2. 18 *September* 1946

Hyperpnoea induced by forced breathing. By **J. N. Mills** . . . 95

The site of pressor action of the base A, closely allied to *l*-nicotine, present in urine. By **Mary F. Lockett** 117

The appearance of a base, detected by a colour test, in the urine of bitches having renal ischaemia. By **Mary F. Lockett** 126

The properties and pharmacological action of a base liberated by alkaline hydrolysis of normal urine, compared with those of a similar base found free in the urine of dogs with renal ischaemia. By **Mary F. Lockett** 138

PAGE

Conduction velocity and myelin thickness in regenerating nerve fibres. By F. K. SANDERS and D. WHITTERIDGE 152

The production and removal of oedema fluid in the lung after exposure to carbonyl chloride (phosgene). By G. R. CAMERON and F. C. COURTICE 175

The absorption of fluids from the lungs. By F. C. COURTICE and P. J. PHIPPS 186

The nucleoproteins of the liver cell demonstrated by ultra-violet microscopy. By J. N. DAVIDSON and CHARITY WAYMOUTH 191

No. 3. 6 *December* 1946

Preliminary observations on the narcotic effect of xenon with a review of values for solubilities of gases in water and oils. By J. H. LAWRENCE, W. F. LOOMIS, C. A. TOBIAS and F. H. TURPIN 197

The metabolism of kidney slices from new-born and mature animals. By MARGERY CUTTING and R. A. MCCANCE 205

Relation between illumination and extent of the visual field. By F. W. SHARPLEY 215

Urea excretion by rabbits. By E. M. VAUGHAN WILLIAMS . . . 223

The effect of adreno-corticotrophic hormone on the respiratory metabolism of the adrenal cortex of the rat. By R. K. CARPENTER, L. D. MACLEOD and M. REISS 231

Maternal and foetal tissue- and plasma-lipids in normal and cholesterol-fed rabbits. By G. POPJÁK 236

On the action of adrenaline on the blood vessels in human skeletal muscle. By W. J. ALLEN, H. BARCROFT and O. G. EDHOLM 255

The experimental production of watery vacuolation of the liver. By O. A. TROWELL 268

No. 4. 15 *January* 1947

The rate and site of acetate metabolism in the body. By D. H. SMYTH 299

The colour sensitivity of the retina within the central fovea of man. By L. C. THOMSON and W. D. WRIGHT 316

The effect of limb position in seated subjects on their ability to utilize the maximum contractile force of the limb muscles. By P. HUGH-JONES 332

PAGE

The ferricyanide method of blood-gas analysis. By F. C. **Courtice** and C. G. **Douglas** 345

Experiments on liver sympathin. By J. H. **Gaddum** and L. G. **Goodwin** 357

The action of chemical vesicants on cholinesterase. By R. H. S. **Thompson** 370

Vitamin A and bone growth: the reversibility of vitamin A-deficiency changes. By E. **Mellanby** 382

LIST OF AUTHORS

PAGE
Adrian, E. D. Components of electroretinogram 24
Allen, W. J., Barcroft, H. and Edholm, O. G. Adrenaline and muscle blood flow 255
Armin, J. and Reeve, E. B. Plasma volume estimations 72
Barcroft, H., Edholm, O. G. and Allen, W. J. Adrenaline and muscle blood flow 255
Beloff, A. and Peters, R. A. Proteolytic activity in burned skin 54
Cameron, G. R. and Courtice, F. C. Lung oedema in phosgene poisoning 175
Carpenter, R. K., Macleod, L. D. and Reiss, M. Corticotrophic hormone and adrenal metabolism 231
Courtice, F. C. and Cameron, G. R. Lung oedema in phosgene poisoning 175
Courtice, F. C. and Douglas, C. G. Ferricyanide method of blood-gas analysis 345
Courtice, F. C. and Phipps, P. J. Fluid absorption from the lungs 186
Cutting, Margery and McCance, R. A. Metabolism of new-born and adult kidneys 205
Davidson, J. N. and Waymouth, Charity. Ultra-violet microscopy of liver 191
Douglas, C. G. and Courtice, F. C. Ferricyanide method of blood-gas analysis 345
Downman, C. B. B. and McSwiney, B. A. Viscero-motor reflexes 80
Edholm, O. G., Allen, W. J. and Barcroft, H. Adrenaline and muscle blood flow 255
Euler, U. S. v. Sympathomimetic substance in heart extracts 38
Gaddum, J. H. and Goodwin, L. G. Liver sympathin 357
Goodwin, L. G. and Gaddum, J. H. Liver sympathin 357
Granit, R. Polarization of retina 45
Gregory, R. A. Intestinal reflexes in nausea and vomiting 58
Hancox, N. M. Occurrence of cells resembling osteoclasts 66
Hebb, Catherine and Nimmo-Smith, R. H. Lung potassium and adrenaline 1
Hugh-Jones, P. Limb position and muscle force 332
Irving, J. T. Dietary restriction and the teeth 17
Lawrence, J. H., Loomis, W. F., Tobias, C. A. and Turpin, F. H. Narcotic effect of xenon 197
Lockett, Mary F. New base in the urine in renal ischaemia 126
Lockett, Mary F. Properties of a base from urine 138
Lockett, Mary F. Site of pressor action of base A 117
Loomis, W. F., Tobias, C. A., Turpin, F. H. and Lawrence, J. H. Narcotic effect of xenon 197

PAGE

McCance, R. A. and Cutting, Margery. Metabolism of new-born and adult kidneys 205
Mellanby, E. Vitamin A and bone cells 382
Macleod, L. D., Reiss, M. and Carpenter, R. K. Corticotrophic hormone and adrenal metabolism 231
McSwiney, B. A. and Downman, C. B. B. Viscero-motor reflexes . . . 80
Mills, J. N. Hyperpnoea after forced breathing 95
Nimmo-Smith, R. H. and Hebb, Catherine. Lung potassium and adrenaline 1
Peters, R. A. and Beloff, A. Proteolytic activity in burned skin . 54
Phipps, P. J. and Courtice, F. C. Fluid absorption from the lungs . 186
Popják, G. Maternal and foetal lipids in rabbits 236
Reeve, E. B. and Armin, J. Plasma volume estimations 72
Reiss, M., Carpenter, R. K. and Macleod, L. D. Corticotrophic hormone and adrenal metabolism 231
Sanders, F. K. and Whitteridge, D. Regenerating nerve fibres . . 152
Sharpley, F. W. Illumination and visual field 215
Smyth, D. H. Acetate metabolism 299
Thompson, R. H. S. Chemical vesicants and cholinesterase . . . 370
Thomson, L. C. and Wright, W. D. Foveal colour sensitivity . . . 316
Tobias, C. A., Turpin, F. H., Lawrence, J. H. and Loomis, W. F. Narcotic effect of xenon 197
Trowell, O. A. Watery vacuolation of the liver 268
Turpin, F. H., Lawrence, J. H., Loomis, W. F. and Tobias, C. A. Narcotic effect of xenon 197
Vaughan Williams, E. M. Urea excretion by rabbits 223
Waymouth, Charity and Davidson, J. N. Ultra-violet microscopy of liver 191
Whitteridge, D. and Sanders, F. K. Regenerating nerve fibres . . 152

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

23 *February* 1946

PAGE

Gibson, Q. H. and Harrison, D. C. The ratios of iron to oxygen, iron to colour and oxygen to colour in the blood of men and women . . . 1*P*
Hartridge, H. Visual acuity of the eye using lights of different colour . 2*P*
Hartridge, H. The repair of the retinal image 3*P*
Hartridge, H. Micro-stimulation of the human retina 3*P*
Hartridge, H. The colour vision obtained by micro-stimulation of the human retina 4*P*
Hartridge, H. Cluster formation by the foveal cones 5*P*
Comline, R. S. (*Introduced by W. Feldberg.*) Synthesis of acetylcholine by non-nervous tissue 6*P*
Bacq, Z. M. and Isola, W. Smooth muscle of the inferior palpebra activated by adrenergic sympathetic nerves 7*P*
Feldberg, W. and Hebb, Catherine. Phosphocreatine and the synthesis of acetylcholine 8*P*
Case, R. A. M. A simple rocking tray for staining histological preparations 9*P*
Bacq, Z. M., Desreux, V., Fischer, P. and Fredericq, Eugène. Simple demonstrations of the reaction of cysteine with chloropicrine and allylisosulphocyanide 10*P*
Kosterlitz, H. W. Dietary protein and histological structure of liver tissue 11*P*
Bowie, J. H. (*Introduced by E. J. King.*) An apparatus for continuous injection of penicillin 11*P*
Whitfield, I. C. (*Introduced by E. W. H. Cruikshank.*) An improved nerve-muscle preparation stimulator for student use 12*P*

3–4 *April* 1946

Barclay, J. A. and Kenney, R. A. An index of renal tubular activity . 14*P*
Stoker, S. Brandon and Pollard, J. (*Introduced by D. Burns.*) Heparin and trauma effects in blood 15*P*
Mills, J. N. The life-span of the erythrocyte 16*P*
Schütz, F. Diuretic action of cyanate 17*P*
Gilding, H. P. and Webb, K. A. Mechanical model of heart and circulation 18*P*
Jacoby, F. Differences in localization, by histochemical means, of alkaline phosphatase within the same organ of different experimental animals 19*P*
Schütz, F. A simple device for recording the action of drugs on the motility of rats 20*P*

11 *May* 1946

Mackay, I. F. S. An experimental analysis of the human jugular pulse tracing 21*P*
Barcroft, J. and Torrens, D. S. The output of the heart of the foetal sheep 22*P*
Floyd, W. F. Effects of temperature and anaesthetics on spontaneous rhythmic contractions of body-wall muscle of the earthworm . . 23*P*
Hill, D. K. Continuous measurement of oxygen concentration in physiological media 24*P*

15 *June* 1946

PAGE

Barcroft, J. and Elsden, S. R. The oxygen consumption of the sheep foetus . . . 25*P*

Euler, U. S. v. Sympathin in adrenergic nerve fibres 26*P*

Knox, J. A. C. A histamine dilatation abolished by atropine 27*P*

Best, C. H., Hoffmann, F., Lucas, C. C. and Talesnik, J. Recent work on the lipotropic factors 27*P*

Barclay, A. E., Daniel, P., Franklin, K. J., Prichard, M. M. L. and Trueta, J. Records and findings obtained during studies of the renal circulation in the rabbit with special reference to vascular short-circuiting and functional cortical ischaemia 27*P*

Barclay, A. E., Daniel, P., Powell, H. M. and Prichard, M. M. L. Radio-micrography: a preliminary communication 28*P*

20 *July* 1946

Thomson, W., Thomson, A. M. and Cuthbertson, D. P. Certain physiological variations in the haemoglobin levels of sheep 30*P*

Harper, A. A. The effect of extracts of gastric and intestinal mucosa on the secretion of HCl by the cat's stomach 31*P*

Davidson, J. N. The effects of fasting on the nucleic acid content of rat tissues 32*P*

Feldberg, W. and Hebb, Catherine. Effect of adenosinetriphosphate and creatine phosphate on the perfused superior cervical ganglion of the cat 32*P*

Campbell, Rosa M. and Kosterlitz, H. W. The influence of sex on liver cytoplasm 33*P*

Marshall, A. Modified van Gieson stain 35*P*

28 *September* 1946

Dewar, A. D., Hall, K. and Newton, W. H. Potentiation of the vaginal response to oestrone by the 'relaxin' fraction of pregnant rabbit serum 37*P*

Willmer, E. N. Observations on the central fovea of certain red-green-blind subjects 38*P*

Hancox, N. M. A method for direct microscopical study of living embryo bone fragments engrafted on the chick chorioallantoic membrane . 39*P*

Gregory, R. A. Application of the London cannula technique to sheep and goats 39*P*

26 *October* 1946

Rogers, A. F. (Introduced by R. L. Brocklehurst.) Arterial spasm in the human umbilical cord 41*P*

Hitchcock, M. W. S. and Phillipson, A. T. The tolerance of sheep to low concentrations of blood sugar 42*P*

Comline, R. S. Effect of flavine-adenine-dinucleotide and other substances on the synthesis of acetylcholine 43*P*

LIST OF AUTHORS

PAGE

Bacq, Z. M., Desreux, V., Fischer, P. and Fredericq, Eugène. Reaction of cysteine with asphyxiant gases 10*P*

Bacq, Z. M. and Isola, W. Adrenergic innervation of inferior palpebra . 7*P*

Barclay, A. E., Daniel, P., Franklin, K. J., Prichard, M. M. L. and Trueta, J. Renal circulation in rabbit 27*P*

Barclay, A. E., Daniel, P., Powell, H. M. and Prichard, M. M. L. Radio-micrography 28*P*

Barclay, J. A. and Kenney, R. A. Index of renal tubular activity . 14*P*

Barcroft, J. and Elsden, S. R. Oxygen consumption of sheep foetus . 25*P*

Barcroft, J. and Torrens, D. S. Heart output of foetal sheep . . 22*P*

Best, C. H., Hoffmann, F., Lucas, C. C. and Talesnik, J. Recent work on lipotropic factors 27*P*

Bowie, J. H. (Introduced by E. J. King.) Apparatus for continuous penicillin injection 11*P*

Campbell, Rosa M. and Kosterlitz, H. W. Sex and liver cytoplasm . 33*P*

Case, R. A. M. Rocking tray for histological staining 9*P*

Comline, R. S. Flavine-adenine-dinucleotide and acetylcholine . . 43*P*

Comline, R. S. (Introduced by W. Feldberg.) Synthesis of acetylcholine by non-nervous tissue 6*P*

Cuthbertson, D. P., Thomson, W. and Thomson, A. M. Haemoglobin levels of sheep 30*P*

Daniel, P., Franklin, K. J., Prichard, M. M. L., Trueta, J. and Barclay, A. E. Renal circulation in rabbit 27*P*

Daniel, P., Powell, H. M., Prichard, M. M. L. and Barclay, A. E. Radio-micrography 28*P*

Davidson, J. N. Fasting and nucleic acid in tissues 32*P*

Desreux, V., Fischer, P., Fredericq, Eugène and Bacq, Z. M. Reaction of cysteine with asphyxiant gases 10*P*

Dewar, A. D., Hall, K. and Newton, W. H. 'Relaxin' and vaginal response 37*P*

Elsden, S. R. and Barcroft, J. Oxygen consumption of sheep foetus . 25*P*

Euler, U. S. v. Sympathin in adrenergic nerve fibres 26*P*

Feldberg, W. and Hebb, Catherine. Phosphates and superior cervical ganglion 32*P*

Feldberg, W. and Hebb, Catherine. Phosphocreatine and synthesis of acetylcholine 8*P*

Fischer, P., Fredericq, Eugène, Bacq, Z. M. and Desreux, V. Reaction of cysteine with asphyxiant gases 10*P*

Floyd, W. F. Contraction of body-wall muscle of earthworm . . . 23*P*

Franklin, K. J., Prichard, M. M. L., Trueta, J., Barclay, A. E. and Daniel, P. Renal circulation in rabbit 27*P*

Fredericq, Eugène, Bacq, Z. M., Desreux, V. and Fischer, P. Reaction of cysteine with asphyxiant gases 10*P*

PAGE
Gibson, Q. H. and Harrison, D. C. Iron, oxygen and colour of human blood . . . 1P
Gilding, H. P. and Webb, K. A. Model of heart and circulation . . . 18P
Gregory, R. A. London cannula technique in sheep and goats . . . 39P
Hall, K., Newton, W. H. and Dewar, A. D. 'Relaxin' and vaginal response . . . 37P
Hancox, N. M. Bone grafts on chorioallantoic membrane . . . 39P
Harper, A. A. Mucosal extracts and secretion of HCl . . . 31P
Harrison, D. C. and Gibson, Q. H. Iron, oxygen and colour of human blood . . . 1P
Hartridge, H. Cluster formation by foveal cones . . . 5P
Hartridge, H. Colours with micro-stimulation of human retina . . . 4P
Hartridge, H. Micro-stimulation of human retina . . . 3P
Hartridge, H. Repair of retinal image . . . 3P
Hartridge, H. Visual acuity with different colours . . . 2P
Hebb, Catherine and Feldberg, W. Phosphates and superior cervical ganglion . . . 32P
Hebb, Catherine and Feldberg, W. Phosphocreatine and synthesis of acetylcholine . . . 8P
Hill, D. K. Continuous measurement of oxygen concentration . . . 24P
Hitchcock, M. W. S. and Phillipson, A. T. Blood sugar in sheep . . . 42P
Hoffmann, F., Lucas, C. C., Talesnik, J. and Best, C. H. Recent work on lipotropic factors . . . 27P
Isola, W. and Bacq, Z. M. Adrenergic innervation of inferior palpebra . . . 7P
Jacoby, F. Species differences in phosphatase distribution . . . 19P
Kenney, R. A. and Barclay, J. A. Index of renal tubular activity . . . 14P
Knox, J. A. C. Histamine dilatation abolished by atropine . . . 27P
Kosterlitz, H. W. Dietary protein and histology of liver . . . 11P
Kosterlitz, H. W. and Campbell, Rosa M. Sex and liver cytoplasm . . . 33P
Lucas, C. C., Talesnik, J., Best, C. H. and Hoffmann, F. Recent work on lipotropic factors . . . 27P
Mackay, I. F. S. Analysis of jugular pulse tracing . . . 21P
Marshall, A. Modified van Gieson stain . . . 35P
Mills, J. N. Life-span of erythrocyte . . . 16P
Newton, W. H., Dewar, A. D. and Hall, K. 'Relaxin' and vaginal response . . . 37P
Phillipson, A. T. and Hitchcock, M. W. S. Blood sugar in sheep . . . 42P
Pollard, J. and Stoker, S. Brandon. (Introduced by D. Burns.) Heparin and trauma effects in blood . . . 15P
Powell, H. M., Prichard, M. M. L., Barclay, A. E. and Daniel, P. Radio-micrography . . . 28P
Prichard, M. M. L., Barclay, A. E., Daniel, P. and Powell, H. M. Radio-micrography . . . 28P
Prichard, M. M. L., Trueta, J., Barclay, A. E., Daniel, P. and Franklin, K. J. Renal circulation in rabbit . . . 27P
Rogers, A. F. (Introduced by R. L. Brocklehurst.) Arterial spasm in human umbilical cord . . . 41P

PAGE
SCHÜTZ, F. Device for recording rat motility 20P
SCHÜTZ, F. Diuretic action of cyanate 17P
STOKER, S. BRANDON and POLLARD, J. (Introduced by D. Burns.) Heparin and trauma effects in blood 15P
TALESNIK, J., BEST, C. H., HOFFMANN, F. and LUCAS, C. C. Recent work on lipotropic factors 27P
THOMSON, A. M., CUTHBERTSON, D. P. and THOMSON, W. Haemoglobin levels of sheep 30P
THOMSON, W., THOMSON, A. M. and CUTHBERTSON, D. P. Haemoglobin levels of sheep 30P
TORRENS, D. S. and BARCROFT, J. Heart output of foetal sheep . . . 22P
TRUETA, J., BARCLAY, A. E., DANIEL, P., FRANKLIN, K. J. and PRICHARD, M. M. L. Renal circulation in rabbit 27P
WEBB, K. A. and GILDING, H. P. Model of heart and circulation . . . 18P
WHITFIELD, I. C. (Introduced by E. W. H. Cruickshank.) Nerve muscle stimulator for students 12P
WILLMER, E. N. Foveal vision of colour-blind subjects 38P

J. Physiol. (1946) 105, 1–15 546.32:577.174.5:612.215.8

THE PARTITION OF POTASSIUM BETWEEN THE ISOLATED LUNG OF THE DOG AND ITS PERFUSATE WITH PARTICULAR REFERENCE TO THE ACTION OF ADRENALINE

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Daly, Hebb & Petrovskaia (1941) described conditions under which it was possible to demonstrate, in the isolated blood-perfused lungs of the dog, the spontaneous reversal of adrenaline action on the bronchial smooth muscle. Adrenaline initially caused broncho-dilatation, but after 2 hr. of perfusion the response obtained was broncho-constriction. Coupled with this was an intensification of the pulmonary vaso-constrictor action of adrenaline. Both the broncho- and vaso-constrictor responses observed after prolonged perfusion were reversed by ergotoxine.

In the course of a study of the adrenaline broncho-constrictor response some preliminary results obtained with the addition of KCl to the perfusate led Daly *et al.* to suggest that the concentration of K in the circuit might influence the type of response produced by adrenaline. Some time ago, during a systematic examination of the blood and plasma K changes which occur during perfusion of dog lungs, we obtained results which could be interpreted to favour this view. The present paper is an account of these results and deals also with the effect of adrenaline on the partition of K between the lung tissue and its perfusate.

METHODS

Experimental. The method of perfusing the isolated right and left lungs separately for the purpose of obtaining two independent preparations from the same animal (one for control and the other for test purposes) has been fully described by Daly *et al.* (1941). To avoid unnecessary repetition we propose to confine ourselves to a brief outline of the surgical procedures (which differ slightly from those employed by the authors cited) together with a fuller description of certain modifications in the apparatus used.

Healthy mongrel dogs of either sex, and 10–16 kg. in weight, were bled to death from the femoral artery cannulated under local anaesthesia (novocaine), after the intravenous injection of 2000–3000 units of Jorpe's heparin. The escaping blood was filtered through glass-wool, previously wetted with isotonic glucose or NaCl solution, and collected in a measuring cylinder with addition of enough heparin to give a final concentration of 1000–1200 units/100 ml. The heparinized blood thus collected was thoroughly mixed, a sample removed for analysis, and the remainder divided into two equal portions with which the two perfusion systems were then filled.

As soon as the animal was dead, the lungs were rapidly dissected out, separated from each other, and weighed. They were then placed in the respiratory chamber on separate enamel drainage pans and each connected, by cannulae in the pulmonary artery and main bronchus, to its own perfusing and ventilating system.

The circulating system for each lung was arranged in the following way. The flow of blood into the pulmonary artery was maintained at constant volume by means of a Dale-Schuster pump. With the pulmonary veins slit open, the venous blood escaped freely from the lung and dripped on to the enamel drainage pan on which the lung rested. The drainage pan consisted of an upper perforated horizontally placed shelf (supporting the lung) held on a narrow curved pan which was tilted sharply and had a spout dipping into a glass collecting reservoir standing on the floor of the respiratory chamber. The arrangement was such that the blood flowed continuously from lung to collecting reservoir and there was no pooling on the pans themselves. From the reservoir the blood was returned directly to the pump (the second reservoir used by Daly *et al.* being omitted), and so to the pulmonary artery once more. The measurements made on the circulation included a continuous recording of the pulmonary arterial pressure (P.A.P.) calibrated in cm. of blood, and readings of the blood temperature and the venous reservoir volume made at 15 min. intervals.

The perfusion was started 30–60 min. after the animal's death, the rates of flow in each lung being adjusted to give an initial perfusion pressure of +15 to +20 cm. of blood (with flow volumes in different preparations of between 200 and 400 ml. per lung per min.). After that, the plate-glass cover with which the respiratory chamber was closed was put into position with an airtight seal of high melting-point vaseline. Each lung was then inflated once, and negative-pressure ventilation begun. The tidal air of each separate preparation was continuously recorded by means of a small spirometer equipped with a writing point. The closed system of lung, spirometer, and intervening airway contained an air volume of 200–300 ml.

In isolated lungs prepared in this way, there is an extensive surface of blood exposed to the continuous air draught in the respiratory chamber as the blood courses over the lungs and shelves of the drainage pans into the venous reservoir below. Unless certain precautions are taken there is a tendency for the blood to lose water by evaporation (Daly, Eggleton, Elsden & Hebb, 1946), and also to become hypocapnic. To avoid these effects, the respiratory chamber was divided by an incomplete partition into two compartments whereby the main sweep of air between the valves and suction apparatus was confined to one compartment while the atmosphere in the other compartment, containing the lungs, was renewed by a controlled stream of a mixture of 5% CO_2 in O_2 saturated with water vapour. With this arrangement we found that: (1) losses of water from the system were negligible; (2) in the absence of any treatment which might induce oedema, the blood chloride and plasma volume remained constant for several hours, thus indicating that there was no spontaneous oedema (cf. Daly *et al.* 1946); and (3) the blood *p*H was maintained between 7·4 and 7·5 (observations in earlier unpublished experiments).

Administration of drugs. Solutions of adrenaline, ergotoxine and ergotamine were injected through a fine needle directly into the pulmonary arterial tubing. Continuous infusion of adrenaline was made through a fine needle similarly inserted into the venous return tubing and connected by pressure tubing to an electrically driven, calibrated syringe. Both adrenaline in solution with chloretone (Parke, Davis and Co.) and crystalline adrenaline (Adrenalina B.D.H.) dissolved in 0·9% NaCl made slightly acid (N/100) with HCl, were used. Since no difference could be detected in the responses to these two preparations no further reference will be made to them individually. Ergotoxine ethane sulphonate (B.D.H.) was dissolved in absolute ethyl alcohol so that, after diluting with 3 parts of 0·9% NaCl solution, 2 mg. of ergotoxine were contained in either 1 or 2 ml. of the dilute solution, as required. Ergotamine tartrate (Sandoz) was dissolved in 0·9% NaCl solution to give a concentration of 1 mg./ml.

Analytical. Plasma K, percentage red-cell volume and haemoglobin determinations were made in all experiments. In some, determinations were also made of whole-blood K, of whole-blood and plasma chloride, and of plasma lactate.

Blood samples were removed from the tubing leading to the arterial cannula; the first 4 or 6 ml. withdrawn were discarded, since there was a small dead space in the T-tube inserted at this point. No precautions were taken against CO_2 loss, but the separation of plasma and the protein precipitation were carried out within the shortest possible time of taking the sample. When plasma was required, blood was centrifuged at 3000 r.p.m. for 10 min. A Krogh-Keys (1931) syringe pipette was found invaluable in making rapid and very accurate deliveries of 1 ml., or less, of blood or plasma.

Potassium was estimated by the argenti-cobaltinitrite method of Breh & Gaebler (1930) as modified and developed for colorimetry by Looney & Dyer (1942). A rigid technique was followed and frequent analyses of standard solutions made; the variations from day to day and even from one batch of reagents to another, however, were found to be very small. The final colour intensity was measured on the Hilger 'Spekker' absorptiometer, using a combination of filters 5 and 6 as supplied with the instrument. The method was found to give satisfactory and reproducible results; for instance, the average difference between duplicates in fifty consecutive analyses of plasma was 2·04%. Estimations were always carried out in duplicate.

Chloride was estimated in trichloroacetic acid filtrates, care being taken to see that the protein precipitant was itself free from chloride. The electrometric titration of Eggleton, Eggleton & Hamilton (1937) was used and, in the later experiments, was modified slightly to allow of the use of smaller aliquots and a more concentrated silver nitrate solution, with consequent increase in sensitivity. Titration was carried out through a 0·2 ml. horizontal burette into a total titration volume of 1·0–1·3 ml. The original method and the modification gave excellent results; duplicate determinations rarely differed by as much as 1%.

For *lactate* Barker & Sumerson's (1941) method was employed, colour intensity being measured by the 'Spekker' using a combination of filters 3 and 5.

The *percentage red-cell volume* was measured in duplicate according to the method described by Meyerstein (1942) for small volumes of blood. An approximate volume of 0·15 ml. was placed in the haematocrit tube and spun for 15 min. at 10,000 r.p.m. Except in the case of blood with an abnormally high sedimentation rate, which presents obvious difficulties of sampling, the differences between duplicates were less than 1%. Samples with a high blood sedimentation rate, which occurred occasionally, sometimes showed differences of the order of 2%.

Blood *haemoglobin* was estimated after its conversion to cyanmethaemoglobin according to the procedure of Stadie (1920) as modified by Wu (1922), the colour intensity being measured by the 'Spekker' with a No. 5 (green) filter. From the readings so obtained, the haemoglobin was calculated by means of a standard curve established from a large series of bloods of predetermined oxygen capacity. The error of measurement, as shown by duplicate determinations, was of the same order as that given for the haematocrit.

The total amount of any plasma constituent was calculated from its determined concentration in the plasma and its total circulating volume, the latter being derived from the known whole-blood volume and the estimated volume of red cells.

In order to facilitate comparison of the amounts of K taken up by or released from the lungs, these exchanges were referred to the initial wet weight of the lung. Previous estimates of the blood content of lungs prepared in a comparable manner (i.e. with opened veins facilitating drainage), carried out by haemoglobin determinations on finely minced lung, revealed that the blood content was of the order of 10% of the total weight (unpublished data). Accordingly, this proportion was deducted from the determined lung weights in making the calculations.

RESULTS

Plasma potassium concentration in control perfusions

It should be remarked at the outset that the blood used for perfusion had a higher K content than when it was circulating in the living animal. The normal values found by Manery & Solandt (1943) for a series of forty-four healthy dogs give an average plasma K value of 15·9 mg./100 ml.; while in the mixed shed blood analysed in our first twelve experiments the corresponding value was 18·0 mg./100 ml. (range 15·8–20·6 mg./100 ml.). This comparison suggested to us that there was an excess of K in the collected blood. In seven later experiments in which we compared the K content of samples taken throughout bleeding we were able to verify this, and found in confirmation of Baetjer's work (1935) that a rise in plasma K occurred owing to the bleeding itself. The results given in Table 1 showed that the terminal K values were from 3·4 to 14·7 mg. higher than those observed at the beginning of bleeding, while in the mixed shed blood the concentration was on the average 3 mg. higher than in the initial sample.

TABLE 1. Changes in plasma K during bleeding and during the first 2–5 min. of perfusion

Bleeding time (min.)	Plasma K (mg./100 ml.)			
	During bleeding		Pooled blood	After 2–5 min. perfusion
	Start	End		
10	15·5	18·9	—	—
6¼	16·1	23·0	17·6	—
9	16·2	26·5	18·2	—
6¾	12·7	27·4	19·4	—
8½	16·2	23·3	18·3	—
5½	15·1	26·2	18·4	
6½	13·8	24·7	16·8	23·5
7½	—	—	12·2	L. 15·2
7½	—	—	12·2	R. 15·7
7	—	—	15·2	L. 17·0
7	—	—	15·2	R. 22·4
8	—	—	19·2	26·5
8	—	—	18·5	31·0
11½	—	—	15·9	L. 21·0
11½	—	—	15·9	R. 22·7

To emphasize the significance which we attach to the increased K content of the blood, let it be supposed that from a 13 kg. dog, 840 ml. heparinized blood, with a cell volume of 40% and a plasma K content of 3 mg./100 ml. above the pre-bleeding level, had been collected. The tissues of the dog thus lost a total of about 15 mg. K into the plasma. Had the lost blood been returned to the animal before the circulation had ceased, the tissues would have again taken up the K gained by the plasma (Thaler, 1935). Assuming that approximately 10 kg. of the dog's tissues were involved in dealing with the extra 15 mg. K, this would represent only 1·5 μg. K/g. tissue. When, however, the whole 15 mg. is to be perfused through the lungs, with a total weight of about 100 g., the proportion will be greatly altered, each g. tissue now being related to 150 μg. K.

At the beginning of perfusion, then, the lungs were being perfused with blood containing a considerable excess of potassium. After 2–5 min. perfusion, that is to say after the whole volume of blood had been carried through the pulmonary vascular bed two or three times, a further increase in the plasma K level was observed (Table 1). The magnitude of this further increase varied.

For example, in one experiment it was equal to 65%, in another it was 17%. These figures correspond to total gains of 29·0 and 5·7 mg. K respectively. The source of this K was undoubtedly the lung tissue itself. Over the same period the K in the blood corpuscles remained constant; this can be stated with some confidence, since the erythrocytes of the dog, as distinct from most other species, contain only very little more K than does the plasma. Thus it was found in analyses of whole blood and plasma that the total changes of circulating K were due to changes in the plasma concentration of the ion. This point is emphasized, since, in the subsequent discussion of our results, it may be thought that the possible effects of changes in corpuscular K have been neglected.

The increase in K could not have been due to contamination because all apparatus was scrupulously cleaned. Further, it was too large to be accounted for by the amount of blood (about 30 ml.) remaining in the lungs of the animal, unless this blood had subsequently had its plasma K content very considerably increased after the end of bleeding. The cessation of blood flow through the lung for the period intervening between death and setting up the perfusion was the probable cause of the increased K. As will be seen further on, we subsequently found that even a 10 min. period of ischaemia caused a considerable rise in blood K.

The uptake of potassium by the lungs over longer periods of perfusion

Curve III in Fig. 1 is typical of the plasma K changes occurring during perfusion for 2 hr. or longer. In this and other similar experiments a steady drop in the plasma K concentration followed its initial rise. The order of the change may be judged from Table 2. For the five experiments shown, the average of the plasma K values found after 1 hr. or more of perfusion under control conditions was 15·1 mg./100 ml., a value which is 2·6 mg. less than the average for the pooled blood before perfusion in the same experiments. In five other experiments, after perfusion for an equally long time under control conditions, the average value was 14·9 mg. K/100 ml. plasma (range 12–18·4 mg./100 ml.). These results indicated therefore that not only were the original losses of K from the lung (at the start of perfusion) made up, but a considerable part of the excess which arose during bleeding found its way into the lung tissue as well.

TABLE 2. Plasma K concentration (mg./100 ml.)

Exp.	Pooled blood before perfusion	After perfusion for 1 hr. or longer (control conditions)
89	18·6	15·5
90	15·8	14·7
91	17·2	16·1
92	20·4	15·7
93	16·6	13·6
Average	17·7	15·1

The fall in the plasma concentration of K did not continue indefinitely but usually ceased during the second hour, rarely earlier (but see Fig. 2 B) or later. Subsequently it was maintained within ±0·5 mg./100 ml. of its minimal level for several hours provided that there was no experimental interference.

The initial rate of fall varied in different experiments, and so it became desirable to know how far the changes observed for each lung of a single pair were comparable. Without this knowledge we could not have the same confidence in evaluating the effects of special procedures which we wished to test.

In order to make such a comparison, we examined the initial changes occurring in the right and left lungs in four experiments in which the blood flow in relation to the weight of tissue was approximately equal for the two lungs and neither had been subjected to any special treatment.

The results of this examination are summarized in Table 3, from which it can be seen that the calculated total uptake of each lung for a given period might vary considerably; the uptake per unit weight of tissue in unit time, however, showed good agreement for any pair of lungs.

TABLE 3. Initial uptake of K by the isolated perfused lung (calculated)

Exp.		Lung weight (g.)	Total gain (mg.)	Perfusion time (min.)	Gain in K (μg./g. lung tissue/min.)
1	L.	65	9·7	27	5·8
	R.	91	17·1	27	7·2
2	L.	65	7·0	14	7·7
	R.	82	7·0	14	6·1
5	L.	45	8·7	23	8·4
	R.	60	12·2	23	8·8
6	L.	45	15·8	32	11·0
	R.	67	23·8	32	11·1

Larger variations in the rate of uptake of K calculated on the same basis in a few experiments, of which that represented in Fig. 2 B is an example, were associated with differences in blood flow. In this particular experiment the volume of blood flow per unit weight of tissue was about 20% larger in the left lung. The calculated uptake of K for each lung after 1¼ hr. was 192 (left) and 196 (right) μg. K/g. lung tissue—a difference which was less than might arise from experimental error. In the left lung, however, this was accomplished within 33 min., but in the right, it took longer.

The migration of K into the tissue and its retention there was dependent upon the maintenance of an adequate circulation. Thus, as mentioned earlier, ischaemia produced by stopping the perfusion pump for 10 min. evoked a rise in plasma K (Fig. 1, curve V, and Table 4). Less prolonged ischaemia (5 min.) was without demonstrable effect. It may be, therefore, that interference with the normal blood supply of the tissue accounted for the fact that in one experiment, where small clots were found in the blood, no progressive decrease in plasma K was observed. Another procedure, gentle inflation of the lungs by positive pressure,

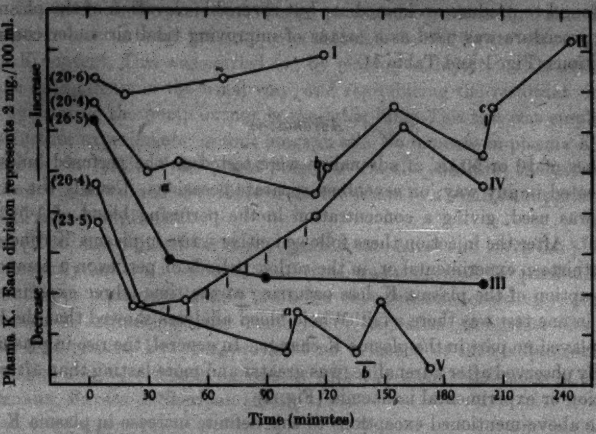

Fig. 1. Curves representing changes in the plasma K concentration of isolated blood-perfused dog lungs. Absolute levels of plasma K obtaining 2–5 min. after the start of perfusion are given in brackets for each curve. I, continuous infusion of adrenaline at 3 μg./min. II, single injections of adrenaline; 10 μg. at *a* and *b*, 50 μg. at *c*. III, typical control experiment. IV, single doses of adrenaline (10 μg.) repeated at 10 min. intervals, as indicated by the signals. V, lung inflated by positive pressure at *a*; ischaemia for 10 min. at *b*.

Table 4. Transfer of K between the isolated perfused lung and its perfusate under various conditions

Exp.	Perfusion time (min.)	Procedure	Total lung K change (mg.)*	Change K (mg./g. lung)*
1 R.	41–111	10 μg. adrenaline each 10 min. (× 8)	−22·4	−246
L.	122	10 μg. adrenaline	− 9·3	−143
	203	5 μg. adrenaline	−12·3	−189
2 R.	120	5 μg. adrenaline	− 5·4	−103
L.	80	2 mg. ergotoxine	− 8·9	−137
	120	10 μg. adrenaline	− 6·8	− 62
3 R.	7–23	Adrenaline infusion 3 mg./min.	+ 1·3	+ 23
	135	10 μg. adrenaline	− 2·0	− 35
L.	7–23	Control	+10·8	+257
	98	2 mg. ergotoxine	− 5·6	−133
	135	10 μg. adrenaline	− 5·4	−128
4 R.	21–123	Control	0	0
L.	21–123	Adrenaline infusion 3 μg./min.	− 1·8	− 29
5 L.	101	Lung inflated	− 2·9	− 65
	135–145	Pump off	− 4·6	−102
R.	100	4 mg. ergotoxine	− 1·5	− 25
R.	138	Lung inflated	− 4·5	−120

* The figures in the last two columns are calculations derived from the known plasma K change, the total volume of plasma in circulation and the initial wet weight of the lung after deducting 10% for the blood.

+ Indicates a gain of K. − Indicates a loss of K.

was found to produce an immediate but reversible elevation of the plasma K This procedure was used as a means of improving tidal air under controlled conditions (Fig. 1 and Table 4).

Adrenaline

Doses of 10 or 50 μg. of adrenaline were tested on the perfused lungs, not pretreated in any way, on seventeen separate occasions. Usually the smaller dose was used, giving a concentration in the perfusing blood of 1·5–2·5 in 1×10^8. After the injection there followed either a rise in plasma K concentration (thirteen experiments) or, in the earlier periods of perfusion a temporary interruption of the plasma K loss occurring at the time (three experiments). Only in one test was there a fall. Whole-blood analyses showed that the blood cells played no part in the plasma K change. In general, the rise in plasma K, usually observed after adrenaline, was greater and more lasting than after lung inflation or experimental ischaemia (Fig. 1).

The above-mentioned exceptions to this definite increase in plasma K were as follows: in one experiment the injection of 10 μg. adrenaline into each lung circulation after 41 min. of perfusion was followed in each case by a barely detectable rise in plasma K; in another experiment a similar injection after 14 min. was not followed by any change in plasma K in the left lung, but there was a definite increase in the plasma K in the right lung; in the experiment already mentioned, in which the perfusing blood was found to contain small thrombi and in which the usual loss of K from the plasma was absent, administration of adrenaline at 128 min. was followed by a relatively small rise in plasma K.

In one experiment of this series (Fig. 1, curve IV, and Exp. 1, Table 4), repeated doses of adrenaline spaced at intervals of 10 min. were accompanied and followed by a relatively very large increase of plasma K, amounting altogether to 22·4 mg. K in 132 min. Some 30 min. after the last injection had been made there was a rapid fall in the plasma K level. In contrast to this the steady infusion of adrenaline at the rate of 3 μg./min. modified the course of the K changes in a much less striking manner (Fig. 1, curve I and Exps. 3 and 4, Table 4). The superimposition of single doses of adrenaline on a continuous adrenaline infusion, carried out on two occasions, was followed in one of them by a small, though definite, additional rise in plasma K and in the other by an indeterminate response.

To summarize the findings, it may be said that, in general, adrenaline increased the plasma K; but the magnitude of the effect for a given dose appeared to be roughly proportional to the lung uptake of K before the test.

Effect of a rise of P.A.P. The action of adrenaline in causing a rise in plasma K might have been due to a specific action of the drug or to some secondary effect. The most obvious relevant change produced by adrenaline, namely, a rise in pulmonary arterial pressure, was therefore tested for its effect on the plasma K content. This was carried out by making an injection of adrenaline into one lung circuit in the usual way, and reproducing the resultant rise in P.A.P. by altering the pump output in the other lung. This test was employed in two further experiments, in four lungs in all. No increase in plasma K was detected as the result of the raised pressure. On the contrary, the increased blood flow was accompanied in three of the four tests by a small but appreciable loss of K from the plasma.

Fenn (1940) has pointed out that K and lactate enter and leave the plasma simultaneously under a number of conditions, and suggested the possibility of K migrating as potassium lactate. In a few experiments, accordingly, plasma lactate and K estimations were made before and after adrenaline administration. The results, however, provided no evidence of such a quantitative relationship, and were thus in agreement with the later conclusions of Fenn, Koenemann, Favata & Sheridan (1940).

Significance of the elevation of plasma potassium

In spite of the fact that the intracellular concentration of K in the lung tissue is of the order of 20 times that of the extracellular fluid and circulating plasma, there can be no doubt that the normal cell membrane is freely permeable to K. The evidence for this belief has been reviewed by Fenn (1940); more recently, the further use of the radioactive isotope K^{42} in the hands of Noonan, Fenn & Haege (1941) has provided strong additional evidence.

In the changes in plasma K concentration described above, direct chemical analysis showed that the blood cells played no part. The extracellular (tissue) fluid, being an ultrafiltrate of plasma, is in Donnan equilibrium with the plasma, having a slightly lower K content than the latter (Greene & Power, 1931; Greene, Bollman, Keith & Wakefield, 1931; Ingraham, Lombard & Visscher, 1932–3), and is thus incapable of acting as a reservoir of K. The alterations in the plasma K level here described represent, therefore, direct transfer of K between the tissue cells and the plasma. Thus a rise in plasma K in our preparations is interpreted as a loss of tissue-cell K, and vice versa.

The increase in plasma K after a 10 min. period of ischaemia is similar to that described by Baetjer (1935) and Fenn, Wilde, Boak & Koenemann (1939) when the hindlimbs of cats were made ischaemic. The release of intracellular K caused by adrenaline, however, cannot be attributed to ischaemia, for under the conditions of these experiments adrenaline produced no reduction in blood flow. The exact mechanism, probably specific for adrenaline, is presumably the same as that involved when adrenaline liberates K from the liver (D'Silva, 1936*a*, *b*; Marenzi & Gerschman, 1937). Whatever the mechanism involved, it can be seen that one common effect is produced by a redistribution of K between plasma and tissue cells, namely, an alteration in the ratio of K inside the tissue cells to that outside (the K_i/K_o ratio).

BRONCHO-MOTOR RESPONSE TO ADRENALINE IN RELATION TO POTASSIUM EXCHANGES

Daly *et al.* (1941) found that the effects of adrenaline on the perfused lungs had a fairly typical time course: it caused broncho-dilatation at the outset of perfusion; 30–40 min. later adrenaline was ineffective and remained ineffective for some 60 min.; and finally between 90 and 120 min. after the start of perfusion a broncho-constrictor response developed. Occasionally the reversal of adrenaline action was more rapid and broncho-constriction could be observed at the beginning of the second hour of perfusion.

In our further observations of the broncho-motor responses to adrenaline we have considered this reversal of its action in relation to the spontaneous changes in the plasma K level which have already been described. We have repeated experiments made by Daly *et al.* (1941), who showed that when adrenaline tests were carried out at the beginning and again 2 hr. after the onset of perfusion, a broncho-dilator or a negative response was caused by the first injection and broncho-constriction by the second.

In our experiments it was possible to show that the early dilator response coincided with high plasma K values and broncho-constriction with relatively low plasma K values. It seemed possible then that the change in the bronchial response might have resulted from this other associated change. A means of testing this theory was provided by several experiments in which for various causes listed below the usual fall in the plasma K concentration had either been prevented or reversed, so that adrenaline tests carried out after 2 hr. or more were combined with relatively high K values. These experiments may be summarized as follows:

(1) Inflation of the lung produced a sharp though temporary increase in the plasma K. When carried out after 2 hr. of perfusion it had the additional effect of temporarily abolishing adrenaline broncho-constrictor responses. Adrenaline injected within the first few minutes after inflation produced broncho-dilatation; if delayed for a further 10–20 min. the injection had no broncho-motor action; but when given 30 min. or more after the inflation it would once again produce broncho-constriction. These conclusions, based on the combined observations of several of our experiments, received further confirmation from a re-examination of the experimental protocols of Daly *et al.* (1941), in which several scattered observations gave evidence of a similar effect of lung inflation, although this was not recognized at the time the experiments were carried out.

(2) Adrenaline infusion was associated with maintenance of initial K values: a single injection of adrenaline superimposed on the infusion at 2 hr. or later caused dilatation (two experiments).

(3) Phosgene poisoning raised the plasma K concentration to levels which equalled or exceeded the initial perfusion values (unpublished data): adrenaline caused dilatation or no response 2 hr. or more after the beginning of perfusion (two experiments).

(4) Failure of the lungs to take up K from the plasma associated with inadequate circulation (experiment earlier referred to in which small thrombi were present in the perfusing blood): adrenaline caused broncho-dilatation.

Thus it seemed that by increasing the plasma K concentration at 2 hr. or later to values approximating to those obtaining in the earliest stage of perfusion we obtained conditions which once again favoured the broncho-dilator action of adrenaline.

TABLE 5. Adrenaline broncho-motor responses of the isolated perfused lung in relation to changes in the distribution of K

Exp.	Perfusion time (min.)	Procedure	Plasma K	Dose of adrenaline (µg.)	Change in tidal air (%)
1 L.	0	(=Unperfused blood)	20·4	—	—
	122	Control	15·9	10	−50
	162	Lung inflation at 143 min.	20·2	10	0
	202		17·8	50	−14
R.	0	10 µg. adrenaline every 10	20·4	—	—
	122	min. from 40 to 100 min.	18·8	10	−22
	162	Lung inflation at 144 min.	23·4	10	0
4 R.	0	Thrombi in circulation	20·3	—	—
	128		21·4	10	+13
	161		22·4	50	+30
95* L.	88	Control	21·0	—	—
	293		18·9	1	−28
R.	88	Phosgene at 72 min.	21·0	—	—
	293		21·1	1	+ 9

It should be noted that the expected broncho-motor response after 90–120 min. perfusion is broncho-constriction.

* The relatively high value for the plasma K obtaining in the control lung of the experiment is due to the two lungs—gassed and control—having been cross-circulated at the time these samples were taken. Subsequently the circulations were separate, and as may be seen the plasma K fell in the control, while it was maintained at the same level in the gassed lung.

The assessment of these experiments had to be based on the changes in the K content of the perfusate relative to its initial concentration. The actual range of values over which adrenaline caused broncho-constriction varied from 12·7 to 18·8 with a mean value of 16·1 mg./100 ml. plasma (seven experiments). Positive broncho-dilator responses were observed with a coincident range of plasma K values of 20·4–23·5 with a mean of 21·9 mg./100 ml. plasma (seven experiments). From this it would appear that broncho-dilatation only occurred under conditions which excluded any uptake by the lungs of the excess K added by bleeding. The lung content of K must certainly have been higher when the opposite response was produced; but it cannot be inferred that broncho-constriction occurred only when the lung uptake of K was maximal. This would obviously be contradicted by an experiment such as No. 1 in Table 5. It should be observed, however, that in this experiment the constrictor action of adrenaline disappeared when the plasma concentration of K equalled or exceeded the value obtained in the unperfused blood (that is, when the lung no longer retained any of the excess added by bleeding), and reappeared when the concentration fell again.

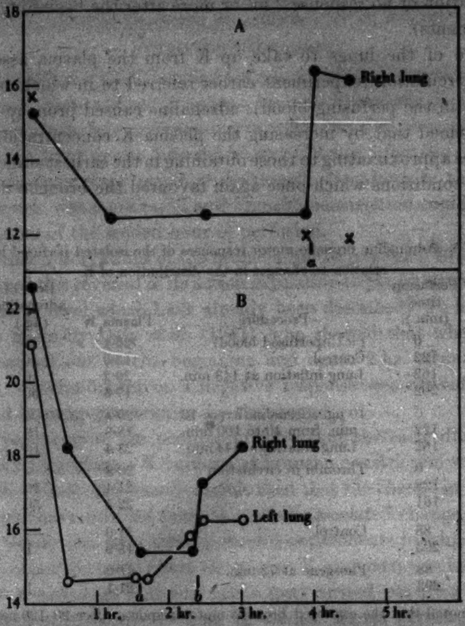

Fig. 2 A. The effect of adrenaline on the plasma K concentration after several hours' perfusion. Abscissa: time in hr. Ordinate: mg. K/100 ml. plasma. The complete curve is for the right lung; the crosses indicate the values in the other lung perfused at the same time. The dose of adrenaline (at *a*), injected into the pulmonary arterial tubing of the right lung, was 20 μg. In addition to the rise in plasma K, adrenaline caused broncho-constriction (tidal air reduction =16%).

B. The effect of adrenaline on the plasma K concentration with and without pre-treatment by ergotoxine. Abscissa: time in hr. Ordinate: mg. K/100 ml. plasma. Injection of 2 mg. ergotoxine into the left lung arterial tubing at *a*; injection of 10 μg. adrenaline into both lungs (same route) at *b*. In the left lung adrenaline caused broncho-dilatation (tidal air increase =50%). In the right lung adrenaline caused broncho-constriction (tidal air decrease =50%).

Note. The difference in the initial rate of fall of plasma K shown by the two lungs is discussed in the text.

Ergotamine and ergotoxine. Broncho-dilator responses to adrenaline were obtained at any stage of perfusion if either of these drugs (doses 1–4 mg.) had previously been injected. The occurrence of such responses under these conditions was not related to any particular change in the K content of the perfusate, which might be low or high relative to the initial level when dilatation with adrenaline was produced. It was also found that after treatment with either of these drugs, the release of K from the lungs by adrenaline was not so rapid (judged by plasma K alteration in 3–5 min.) as was found in control lungs tested at the same time (see Fig. 2). The total amount of K released from the lungs over periods as long as 20–30 min. after an adrenaline injection might in some cases be of the same order as was found in control lungs (see Table 4).

DISCUSSION

The present investigations were undertaken largely as a result of the observation made in another connexion that freshly prepared lungs possessed a remarkable capacity for removing K from the perfusate. In considering the explanation of this phenomenon we realized that the initial blood K values were higher than normal, and we supposed that the shift of K into the lung occurred because of this. Such an effect might be expected, since the incorporation of K into the tissues would tend to restore the normal K_i/K_o ratio. Part of the excess, however, is derived from tissues other than the lung, and the fact that nearly all of this fraction disappears from the blood before the plasma concentration is stabilized indicates that equilibrium is achieved with the lung intracellular K values becoming higher than normal. This conclusion does not mean that the K_i/K_o ratio is above the normal. If the extra K were to be distributed according to that ratio (which is probably about 20 : 1) the plasma would only retain about 5% of it, and we should find, as we do, that the minimal perfusion values approximate to the normal values. That is, the difference would be too small to be appreciated except in a very large series of determinations.

Observation of the spontaneous uptake of K by the perfused lung led us to investigate the possibility that this change is responsible for the altered direction of the bronchial response to adrenaline after prolonged perfusion. The evidence which we have cited strongly suggests that the two phenomena are closely related. On the other hand, the correlation of relatively high tissue K values with the occurrence of adrenaline broncho-constrictor responses is not in itself sufficient evidence that the one effect is the cause of the other. It would agree equally well with the observed facts if we assume that the relation is an indirect one and that each effect is a separate manifestation of some other more fundamental change in the tissue. The information we obtain from these experiments does not therefore provide a clear solution of the problem of the spontaneous reversal of adrenaline broncho-motor action, and to that extent the results are somewhat disappointing. We believe, however, that the experiments are sufficient evidence that the K distribution between the lung and blood has some real significance in relation to the reactivity of the bronchial muscle, and that the elucidation of the problem may be greatly helped when we obtain more knowledge of the mechanisms which govern the K exchanges which occur under the conditions we have described.

A more clear-cut result is the finding that adrenaline may cause the release of K from the lungs. It would be interesting to know whether a similar response occurs in the living animal. The role played by the liver in the increase of plasma K which occurs with injection of adrenaline into the whole animal has received considerable attention (D'Silva, 1934*a*, *b*, 1937), but, so far as we are aware, no critical evidence exists to show that the lungs do not participate in this response. It may be that the effect which we have seen depends upon the special conditions of experiment; and for that reason we do not regard our own results as sufficient evidence that the release of K from the lung is a normal response to adrenaline.

SUMMARY

1. Changes in the partition of potassium between the tissue cells of the isolated perfused lungs of dogs and the plasma of the perfusing blood have been investigated and have been found to follow a definite sequence under standard conditions.

2. Blood used in these perfusion experiments has a higher plasma K content than circulating blood. During the first few minutes of perfusion the plasma K is further augmented by K escaping from the lung tissues.

3. Thereafter there follows a progressive transfer of K from the plasma to the lung-tissue cells, whereby the pre-perfusion level, or an even lower level, is reached in about 60–120 min.

4. Gentle inflation of the lungs by positive pressure, and ischaemia produced by shutting off the arterial pump, both cause a reversible elevation of the plasma K of relatively short duration.

5. Under certain conditions adrenaline injected into the circulation causes a definite increase in the plasma K level. Continuous infusion of adrenaline has much less effect. Repeated doses of adrenaline produce the greatest increase of plasma K. The effect is not secondary to a raised pulmonary arterial pressure.

6. There exists a correlation between the relative concentration of K in the plasma and the response of the pulmonary smooth muscle to adrenaline. A relatively low plasma K level favours adrenaline broncho-constriction, and factors which raise the plasma K level or interfere with its initial fall (inflation, ischaemia, phosgene poisoning, previous treatment with adrenaline) also tend to favour broncho-dilatation.

7. The occurrence of adrenaline broncho-dilatation in ergotoxine- and ergotamine-treated lungs provides an exception to this correlation, since, with such treatment, dilator responses may be associated with low plasma K values.

Our thanks are due to Prof. I. de Burgh Daly for his friendly interest and valuable criticism throughout this research. We are also indebted to the Earl of Moray Fund of Edinburgh University for grants which have defrayed part of the costs of the investigation. We have to thank the Ministry of Supply for permission to publish the results of certain experiments.

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THE EFFECT OF DIETARY RESTRICTION UPON THE INCISOR TEETH OF RACHITIC RATS

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(*Received* 30 *November* 1945)

In previous papers (Irving, 1944, 1946), a description was given of the effects of vitamin D, and of altering the Ca : P ratio of the diet, upon the incisor teeth of rachitic rats. It was found that the administration of phosphate to rats on a high Ca : P ratio type of rachitogenic diet caused quite a different response in the teeth from that occurring after vitamin D dosage. Since starvation is well known to cause spontaneous healing in rachitic animals (McCollum, Simmonds, Shipley & Park, 1922; Coward, 1938), experiments were undertaken to investigate the effect of such a procedure on the tooth, and are reported in the present paper. A preliminary account of part of this work has been already published (Irving, 1945). In view of the findings of Cavins (1924), Wilder (1929) and Kramer, Shear & Siegel (1931) that the blood Ca falls and the P rises during starvation of rachitic animals, and also when P is added to a high Ca : P ratio diet, it was anticipated that the changes in the teeth of starved rachitic animals would be the same as those after addition of P to the diet, and this, in fact, was actually found. Since this work was completed, Schour & Massler, in a review article (1945), mentioned unpublished work by Weinmann & Schour, in which starvation of rachitic animals produced 'a healing effect in the dentin', but no further details were given. The results of dietary restriction on rats having diets with a low Ca : P ratio are also reported; they were quite different from those found when Ca was added to such a diet, hardly any change at all occurring in the teeth.

METHODS

Animals. As in the previous experiments, young albino rats of the Wistar Institute strain were used, in litters of nine, eight or six. The conditions of housing were unchanged. As some difficulty had been experienced in getting marked rickets, the mother and young animals were given, after the young were 14 days old, a modified stock diet poorer in vitamin D than the stock diet regularly used. The young rats were weaned at 23 days of age, and, when they weighed from 50 to 60 g., were transferred to one of the experimental diets on which they were kept for a further 28 days.

Diets. Two experimental diets were used, similar to those previously described. Their composition is given in Table 1; they are referred to in the text by their Ca : P ratio number. Diet 5·6 was the

Steenbock & Black (1925) rachitogenic diet No. 2965. Diet 0·26 was a modification of the Steenbock & Black diet made by reducing the $CaCO_3$ and adding appropriate amounts of Na_2HPO_4 anhyd. (Merck, *puriss.*).

The diets were given slightly moistened with water. When vitamin D was administered, it was in the form of irradiated ergosterol in oil (the international standard preparation), and was given in one dose by mouth, using a micrometer syringe.

Treatment of the animals. Eight litters, three of nine rats, three of eight rats and two of six rats, were used. After 28 days on the experimental diet, one animal was killed as a control. In the case of the nine- and eight-rat litters, three rats were dosed with 27 i.u. of vitamin D, and continued on the experimental diet, in order to compare the effect of the vitamin with the other changes observed. The other five or four rats were also continued on the experimental diet, but their food intake was restricted so that they would lose about 3 g. in weight per day. During the period of dietary restriction they were housed in cages with no bedding and raised about 4 in. above the trays, so that the faeces dropped through and could not be eaten. The experiment was planned so that one dosed and one restricted rat would be killed on the 2nd, 4th and 6th days after dosage or dietary restriction, the remaining restricted rats being killed on the 8th, and in the case of the nine-rat litters, 10th day. The plans for the treatment of the six-rat litters were similar. One rat was killed as a control and two dosed with vitamin D. The food intake of the remaining three was restricted. In one case (animals on diet 5·6) the dosed animals were to be killed on the 4th and 6th days and the restricted ones on the 1st, 2nd and 4th days; in the other case (animals on diet 0·26), the dosed animals were to be killed on the 2nd and 4th days and the restricted ones on the 2nd, 4th and 6th days. In certain cases, however, these plans could not be followed, owing to the deterioration in the condition of the restricted rats.

TABLE 1. Composition of the experimental diets

Ca : P ratio	Ca %	P %
5·6	1·39	0·25
0·26	0·098	0·37

Weights of the rats. The rats were weighed weekly during the initial 28 days on the experimental diets, and daily during the experimental procedures described above.

Examination of the rats. Longitudinal sections of the upper incisors were examined in the way previously described (1944), and the same method of computing the time of action of the various procedures was used. The line test was applied to the lower ends of the radius and ulna (Coward, 1938), and histological examination was made of some of the bones by the methods previously employed (Irving, 1946). In some instances the serum Ca was estimated by the method of de Loureiro & Janz (1944).

RESULTS

Animals on diet 5·6

Two litters of eight rats and one of six rats were used. The animals dosed with vitamin D maintained their weights or slowly gained, as is usual with this diet. The restricted rats lost on an average 3·5, 3·3 and 5·0 g./day in the three litters respectively. They all appeared to be on the verge of tetany, which is well known to happen when rachitic rats are starved. Two animals died, one after 6 days and the other after 4 days of dietary restriction.

In two litters the line test became positive in the restricted animals after 2 days, and in the third after 4 days. As has been already found by McCollum *et al.* (1922) and others, the response in the epiphysis was, in almost all cases, indistinguishable from that seen after vitamin D medication (Pl. 1, figs. 1, 2).

In some cases the line was rather nearer the bone on the epiphyseal side of the cartilage than is found after vitamin D; histological study showed this to be due to the relatively larger metaphysis, the new calcification occurring in the usual site, the junction of the cartilage and the metaphyseal osteoid. Over the period of the experiment, stages of healing up to '3' occurred. The usual epiphyseal healing took place in the animals dosed with vitamin D, being first found on the 4th day after dosing in all litters. Kramer *et al.* (1931) also observed that starvation caused an earlier epiphyseal response than vitamin D. Once healing had begun in the dosed rats, the rate of healing seemed to be about the same as in the restricted animals over the period of the experiment. The epiphyseal response after addition of P to the diet has been found to be quite different from that described above. Usually no line was formed and calcification began as upgrowths of calcified material from the diaphysis into the metaphysis (Irving, 1946).

The teeth of the animals receiving vitamin D reacted shortly after dosage, and the histological appearance was the same as that previously described (Irving, 1944) (Pl. 2, fig. 3). The changes in the teeth after dietary restriction were also visible after 1 day, and calculation showed that the effect of the restriction must have begun to act within a very few hours. These changes were, however, quite unlike those caused by vitamin D and were exactly the same as those found when phosphate was added to the diet (Irving, 1946) (Pl. 2, fig. 4). The first change seen was a fine hypercalcified line on the lingual side and in the distal part of the labial side, affecting the most recently formed dentin. Subsequently there was a disorderly building up of large calcospherites from the old calcified dentin towards the odontoblasts, in the intermediate and proximal labial predentin, the distal stripe widening, and extending backwards and blending with the calcospherites. There was no fine calcospherite response, leading up to a new calcified line, as with vitamin D.

Measurements of the reactive dentin showed that the responses to vitamin D and to dietary restriction must have begun in the tooth after at most 6 hr. As time went on, however, the reaction to vitamin D became more marked than that to dietary restriction, which, while advancing, was going at a slower speed.

Thus after dietary restriction, the changes in the bones were like those following vitamin D medication, but quite unlike those seen after addition of phosphate to the diet. In the teeth the opposite was found.

Animals on diet 0·26

Five litters of animals were used, three of nine rats, one of eight and one of six. The animals dosed with vitamin D showed quite large increases in weight over the experimental period. The restricted rats lost on an average 3·5 g. in weight per day; the highest average daily loss per rat was 4·6 g. in one litter, the lowest being 2·1 g. The restricted animals showed no ill-effects from the restriction provided this was not too extreme. On the other hand, the animals on diet 5·6 all looked unwell. If, however, the daily loss exceeded about 4 g., then the animals deteriorated after 4 days. One was found dead on the 5th day and four had to be killed at this time as they were almost moribund. In view of this, the corresponding rats dosed with vitamin D were also killed 5 days after dosage. The appearance of the moribund rats was quite different from that of rats on diet 5·6. They had no signs of tetany, though their serum-Ca values were all low. They were almost comatose, with extreme weakness and inco-ordination.

The bones of the animals on diet 0·26 did not show rickets, and, as is usual with low Ca : P ratio diets, the epiphyseal cartilages were narrow (Pappenheimer, McCann & Zucker, 1922; Boyle & Wesson, 1943). The width of the epiphyses did show some variability, some being greater, like stage 5 in the conventional scale of healing (Coward, 1938). However, no relationship appeared to exist between the appearance of the bones and the treatment of the animals, and it was therefore assumed that this was a natural variation.

The action of vitamin D on the incisors was the same as that previously described (Irving, 1944); the teeth responded within a day of dosage and reacted strongly and continuously over the period of the experiment. The first signs were hypercalcified stripes on the lingual and distal labial sides, followed by the interglobular dentin response in the intermediate and proximal predentin.

The teeth of the restricted animals behaved in quite a different way, and the changes that occurred were very slight and found only on the lingual and distal labial sides. After 2 days in one litter, 4 days in three litters and 8 days in one litter, very faint signs of recalcification were found on either the labial side or both sides in the most distal part of the tooth. In some cases, recalcification appeared as lines of very fine calcospherites laid down along the sides of the dentin tubules through the whole width of the predentin. In other cases, this appearance was present but, in addition, the fine calcospherites had fused together in the most recently formed predentin to form a very narrow hypercalcified stripe (Pl. 2, fig. 5). The very fine lines of calcification in the dentin tubules lay between the hypercalcified stripe and the already calcified dentin. The formation of this hypercalcified stripe was not, however, invariable. On the lingual side this phenomenon extended farther back with time; in one case it affected the newly formed predentin on almost the whole of the lingual side by the 5th day, in others it took 8–10 days to do this. On the labial side it became more extreme but never extended back very far. As the hypercalcified stripe extended backwards on both sides, it was sometimes preceded by the calcification in the tubules; at other times both types of calcification happened simultaneously. The hypercalcified stripe became very little wider over the period of the experiment, and even when intensely calcified could still be seen to be composed of masses of tiny calcospherites, a condition unlike that found in normal calcification.

This type of calcification sometimes occurred in the control teeth, and thus it was difficult to decide in the early stages whether it was a real response to dietary restriction or not. In the later teeth, however, the calcification extended farther back than in the control teeth. But in no case, even up to 10 days' restriction, was anything seen in the proximal and intermediate labial predentin, where the most striking changes occurred after vitamin D medication. The slightness of the whole response was very remarkable when compared with that caused by vitamin D or with that found when animals on diet 5·6 were restricted.

Serum-Ca estimations were carried out on the last four litters on diet 0·26. The results are summarized in Table 2. In five cases it was impossible to get blood for analysis from the restricted animals. These figures show that vitamin D caused a rise in blood Ca which reached its maximum in 4 days and then declined. No such change occurred with dietary restriction. Here the blood-Ca figures, while showing some small variations, tended, if anything, to fall as the experiment proceeded.

TABLE 2. Serum-Ca values (mg./100 c.c.) of rats on diet 0·26 dosed with vitamin D or restricted in dietary intake for the number of days stated

	Dietary restriction				Dosed with 27 i.u. of vitamin D			
Days Litter no. ...	740	745	801	784	740	745	801	784
0	5·3	5·7	5·6	7·6	5·3	5·7	5·6	7·6
2	4·2	6·1	6·3	6·3	6·7	8·2	7·5	9·2
4	5·7	5·5	4·0	5·7	8·0	10·4	8·1	9·6
5	4·2	—	—	—	6·3	9·4	—	—
6	—	—	—	6·0	—	—	7·5	—
8	—	—	3·6	—	—	—	—	—
10	—	—	5·4	—	—	—	—	—

DISCUSSION

Cavins (1924), Wilder (1929) and Kramer *et al.* (1931) found when rachitic animals on diets like No. 5·6 were fasted, that the blood Ca fell and the P rose. Kramer *et al.* found similar changes when P was added to the diet. It was therefore to be expected that the response in the teeth would be the same with the two procedures. The tooth reaction appears to be fairly specific to the chemical changes in the blood, since after vitamin D, when the blood Ca and P both rise (Kramer *et al.*), the tooth reacts quite differently (Irving, 1944). No adequate explanation, however, is at present forthcoming of the differences in the changes in the epiphyses after dietary restriction and after addition of P to the diet.

No reference has been found in the literature to studies of the effects of starvation on animals subsisting on low Ca : P ratio diets such as diet 0·26. The changes in the teeth, though slight, were of a nature not observed before in any experimental procedure. As stated above, very early changes of the same kind were sometimes seen in the teeth of unstarved animals on diet 0·26, and thus may possibly be attempts at calcification carried out by the odontoblasts under such adverse dietary conditions. During dietary restriction, conditions for such a type of calcification might improve. The fact that the changes in the teeth were so slight led the writer to investigate the blood-Ca level.

As shown in Table 2, the serum-Ca figures remained almost constant when the diet was restricted, though they rose when vitamin D was given. The work of Wilder (1929), in particular, probably explains why the values did not change during dietary restriction. He showed that, during starvation in animals made rachitic by a high Ca : P ratio diet, the P which was mobilized came from the soft tissues and that there was a concomitant depression of the blood Ca. With diets such as No. 0·26, a similar mobilization of soft tissue P presumably also occurred during dietary restriction, and so the blood Ca was kept low and might in fact sink slightly lower. This unaltered blood picture would explain why the teeth reacted so slightly. It seems remarkable, however, that no mobilization whatever of Ca occurred over an interval as long as 10 days, when as much as 37% of the body weight could be lost.

When animals on diets like No. 5·6 are starved, it is well known that they go into tetany (Cavins, 1924; Wilder, 1929). It is at first sight difficult to understand why the animals on diet 0·26 did not have tetany either before or during dietary restriction, when, as with animals on diet 5·6, the blood Ca was presumably being depressed by the liberation of P from the soft tissues. Many workers have found low blood-Ca values in rats with no signs of tetany at all, when using low Ca diets. Thus Templin & Steenbock (1933) observed figures as low as 4·8 mg./100 c.c. serum with no tetany, and Boelter & Greenberg (1941) have recorded similar findings. The probable explanation is that the total blood Ca is not as important in this respect as the ionized Ca. Injections of NaF into animals on diet 0·26 immediately cause tetany (Irving, 1943), presumably by lowering the ionized fraction of the blood Ca.

A comparison of the blood-Ca changes with the reactions in the teeth of rats on diet 0·26 after vitamin D dosage shows one interesting point. The blood Ca reached its maximum in 4 days and then declined. The teeth, however, continued to react strongly, even for as long as 10 days (Irving, 1944). This finding would appear to support the conclusions of Greenberg (1945) drawn from studies in which radio-Ca was used, that vitamin D acted both on Ca absorption and also directly on the calcifying structures.

The amount of weight lost before coma or death in the restricted animals on both diets varied from 24 to 30%, though one animal on diet 0·26 lived for

10 days, losing 37% body weight. This amount of weight loss before fatal results ensued is slightly less than that found by Osborne & Mendel (1917) in normal rats of about the same weight. It would be expected that stunted animals on abnormal diets would not survive large losses of weight so well, especially when, as in the case of those on diet 5·6, they soon went into a tetany-like condition.

SUMMARY

1. Young rats weighing from 50 to 60 g. were placed for 28 days on either the Steenbock & Black rachitogenic diet No. 2965, or else on a modification of this diet with a low Ca : P ratio. They were then either given one dose of vitamin D (27 i.u.) or their food intake was restricted, so that a daily loss of body weight of about 3 g. occurred. They were killed at intervals, and their bones and incisor teeth were examined. Serum-Ca estimations were made in some instances.

2. Animals on the rachitogenic diet with a Ca : P ratio of 5·6, whose food intake was restricted, had a positive line test in their epiphyses after 2–4 days, which was indistinguishable from that caused by vitamin D. The teeth showed a calcification reaction within a few hours of dietary restriction. This response was not like that caused by vitamin D but was the same as that which occurs when P is added to a high Ca : P ratio diet. The restricted animals all had tetany-like symptoms.

3. Animals on the diet with a Ca : P ratio of 0·26 had epiphyseal cartilages of almost normal width, though their teeth were malcalcified. They showed either no changes in their teeth during dietary restriction, or else a very slight and unusual type of recalcification. The low serum-Ca values of these animals were either unchanged during dietary restriction, or else fell very slightly farther. These rats had no signs at all of tetany.

4. The serum-Ca values of rats given vitamin D on the low Ca : P ratio diet rose to a maximum in 4 days and then declined towards the original figures. The recalcification of the teeth caused by vitamin D, on the other hand, proceeded continuously over the whole duration of the experiment.

The writer is indebted to Mr M. W. P. Nienaber for carrying out the serum-Ca estimations; to Prof. R. Goetz and to Mr J. W. Bates for taking the microphotographs; to Dr H. M. Schwartz for assistance with the dosing of the animals; and to Mr D. G. Duncan for technical assistance. The expenses of this work were defrayed by grants from the Staff Research Fund, University of Cape Town, and the National Research Council and Board, Union of South Africa; for these grateful acknowledgement is made.

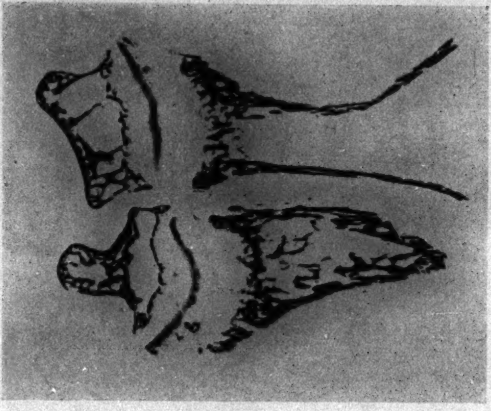

Fig. 2.

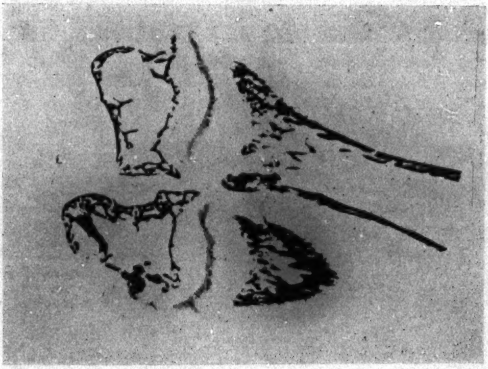

Fig. 1.

To face p. 22

Fig. 5.

Fig. 4.

Fig. 3.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1 and 2 are longitudinal frozen sections of the lower ends of undecalcified ulnae and radii, treated with $AgNO_3$. Magnification ×11·3.

Fig. 1. Line-test response in a rat on diet 5·6, given 27 i.u. of vitamin D 4 days previously.

Fig. 2. Line-test response in a rat on diet 5·6 whose dietary intake had been restricted for 6 days. The response is very similar to that caused by vitamin D.

PLATE 2

Figs. 3–5 are longitudinal sections of the labial side of upper incisor teeth. *a* = dentin, *b* = predentin, *c* = odontoblasts. Magnification ×136.

Fig. 3. Section from an animal on diet 5·6 for 28 days and then dosed with 27 i.u. of vitamin D and killed 4 days later. Intermediate part of the tooth, showing the typical reaction to vitamin D in the predentin.

Fig. 4. Section from an animal on diet 5·6 for 28 days and then subjected to dietary restriction for 2 days. Intermediate part of the tooth, showing the interglobular dentin response in the predentin.

Fig. 5. Section from an animal on diet 0·26 for 28 days and then subjected to dietary restriction for 10 days. Extreme distal part of the tooth, showing the calcified line in the predentin and the calcification running up the dentin tubules in the older parts of the predentin.

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ROD AND CONE COMPONENTS IN THE ELECTRIC RESPONSE OF THE EYE

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(*Received* 12 *December* 1945)

The electric response of the human eye to a flash of light can be divided into a rapid and a slow component (Adrian, 1945); the former is present in the light-adapted eye and is more prominent with light of longer wave-length, the latter is more prominent after dark-adaptation and with the shorter wave-lengths. The obvious suggestion is that the rapid component is related to the activity of the photopic receptor mechanism and the slower to that of the scotopic. As the electric response of the eyeball is in any case a complex affair to which several processes may contribute, it seemed advisable to test the hypothesis by examining the eyes of other species. Records have therefore been made from various animals including monkeys and pigeons, the monkey being chosen because its vision comes nearest to that of man, and the pigeon because it has good colour vision and an eye with many cones. The dual type of response has been found in both these eyes. On the other hand, it has not been found in the eyes of cats, rabbits and guinea-pigs where rods predominate. Records from the optic nerve in these animals have cleared up some doubtful points.

THE ELECTRIC RESPONSE OF THE EYEBALL

Method

The method was as far as possible a copy of that used for the human eye, the chief point being the large retinal area which was illuminated by the flash of light. As a rule the animal was lightly anaesthetized with a barbiturate, the cornea was treated with novocain and the pupil dilated with atropine. The head was placed so that the eye was close to the centre of an opal glass bowl, 12 in. in diameter, with its concave surface towards the eye. The convex surface was illuminated by a beam from a 1000 c.p. pointolite lamp after passage through neutral and Ilford or Wratten monochromatic filters. A wooden sector disk gave flashes of varying duration and frequency. Each flash was signalled by a photoelectric cell connected with one channel of a Grass ink-writer oscillograph; the electric response of the eye was recorded by another of the channels, using the longest time constant available with the amplifier (decay time to half maximum 0·4 sec.). The input leads were connected, one with a moist thread electrode making contact with the cornea, and the other with a moist pad on the ear or the skin near by. Details of the apparatus and curves showing the performance of the monochromatic light filters are given in the previous paper.

Results

Eyes with many cones

Man and monkey. Fig. 1 summarizes the results previously obtained with the human eye, showing the brief wave which is all that can be obtained with a flash of deep red light and the slower component which appears with every colour but red when the eye is moderately dark-adapted. The two components can be distinguished most clearly in the response to an orange-red screen which excludes wave-lengths shorter than 610 mμ. If we are right in thinking that these two forms of response are related to the photopic and scotopic receptor mechanisms respectively, we should expect to find the brief type more prominent in animals with mainly diurnal vision and the slow type in those with mainly nocturnal. And both should be present in eyes which can be used, as in man, either for day or night vision.

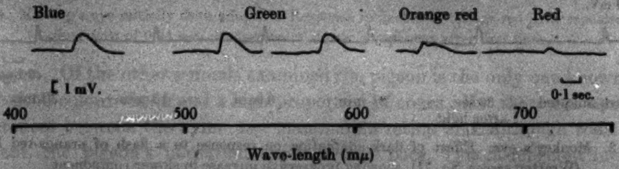

Fig. 1. Electric response of human eye to flashes of light of different wave-length, showing brief ('photopic') response with red, long ('scotopic') response with blue, and double response with orange red. Subject E.D.A. Duration of flashes 0·04 sec. Brightness of screen (in e.f.c.) 1·3 for blue, 1·3 for green, 3·5 for orange red, 3·3 for red. Wratten monochromatic screens 76–70. Upward movement indicates cornea positive to back of eye.

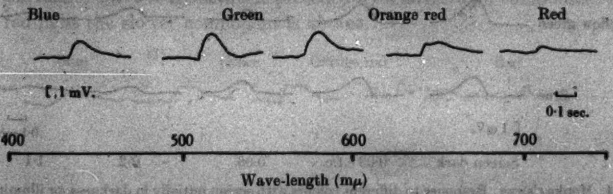

Fig. 2. Response of monkey's eye to flashes of different wave-length. Monkey under light nembutal anaesthesia, pupil dilated with atropine. Brightness of screen as in Fig. 1.

The eye of the monkey certainly behaves like that of man, giving the brief response with red light, the slow with blue and the compound with intermediate wave-lengths. Fig. 2 gives a series of records from a *Rhesus* monkey with the eye moderately dark-adapted. The dual character of the response to orange red is again quite clear: in fact, the only difference from the human records is that in some monkeys there is a trace of the slower response even with red light if the eye is fully dark-adapted.

As in man, the fact that the form of the response varies in this way with the wave-length might mean very little if it had only been found with light of a particular intensity. With the apparatus in use it has not been possible to test the monkey's eye over a very wide range, but a twentyfold variation in intensity for red light and a hundredfold for blue leaves the form unaltered, and a reduction in the intensity of red or orange-red light has never caused any lengthening of the response. As in man, dark-adaptation leads to a great increase in the slower component. This is shown in Fig. 3, which gives the responses to an orange-red flash before, and at various times after, exposure to bright light, and in Fig. 4, which illustrates the effects of flashes of different colour thrown on a screen initially in darkness, or illuminated by white light of different intensities. As the initial illumination is raised, the slow com-

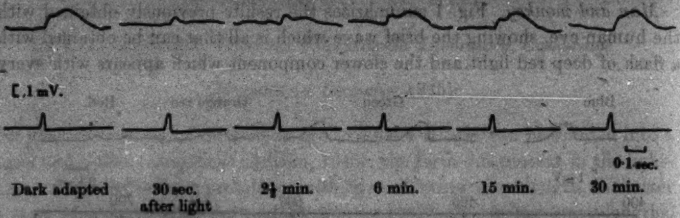

Fig. 3. Monkey's eye. Effect of dark-adaptation on response to a flash of orange-red light (Wratten screen No. 71), showing progressive increase in slower component.

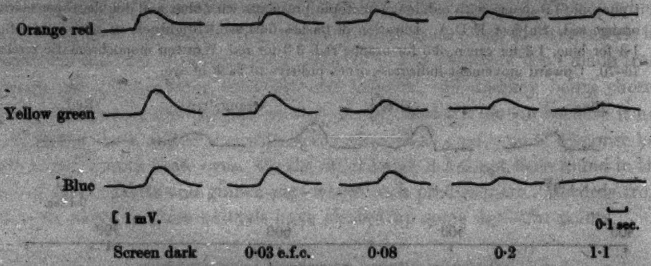

Fig. 4. Monkey's eye. Response to different colours with screen initially in darkness, or illuminated by white light of different intensities, showing reduction of slower component as initial illumination is increased.

ponent becomes smaller and smaller, but the brief component is very little affected. The reduction of the slower component is illustrated in another way in Fig. 5, where the eye is initially dark-adapted and is exposed to orange-red flashes repeated at short intervals.

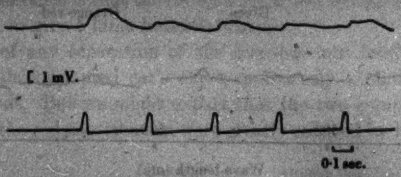

Fig. 5. Monkey's eye initially dark-adapted. Response to flashes of orange-red light repeated at intervals of 0·34 sec. Reduction of slower component after first flash.

Pigeon. Of the other animals examined the pigeon is the only one known to have good colour vision and a high proportion of cones. But there are various differences between the avian and mammalian retina and, although rods are present, the pigeon's nocturnal vision is very poor. This is reflected in the greater prominence of the rapid component and in the longer time needed for dark-adaptation to bring out the slow component. Records from two pigeons are given in Fig. 6. Both had been kept in darkness for some hours before the experiment. A flash of blue light gives a small response of the slower type, but with the longer wave-lengths this is preceded by a large rapid component, and with red light the slower component is absent.

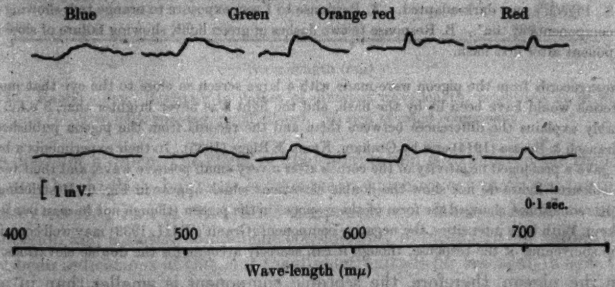

Fig. 6. Pigeon's eye. Response of two eyes to flashes of different wave-length. Birds under nembutal anaesthesia, pupils dilated with atropine. Eyes dark-adapted. Brightness of screen as in Fig. 1.

Fig. 7 shows the effect of a short period of light-adaptation. The slower component is now absent with all wave-lengths, but the rapid component is still large. The disappearance of the slow component can be seen in Fig. 8B, where the eye had been in darkness before the record was taken and was then exposed to flashes of green light repeated at short intervals. In Fig. 8A the exposure (to orange red) was continued for a second: since the slow component appears in its usual position it is clear that it is due to the illumination and is not an 'off' effect.

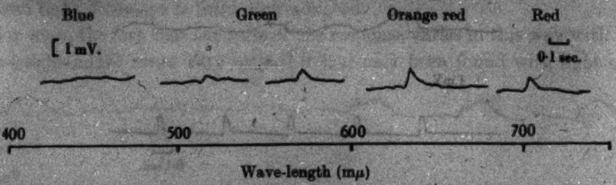

Fig. 7. Pigeon's eye, light-adapted. Response to different wave-lengths. Brightness as in Fig. 1.

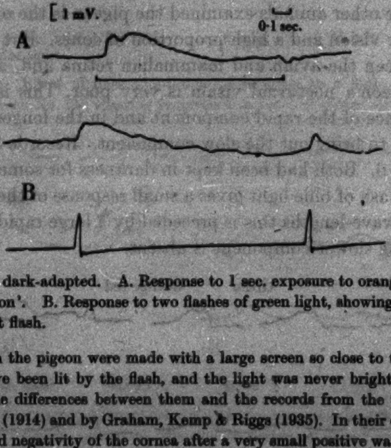

Fig. 8. Pigeon's eye, dark-adapted. A. Response to 1 sec. exposure to orange red, showing both components at 'on'. B. Response to two flashes of green light, showing failure of slow component after first flash.

These records from the pigeon were made with a large screen so close to the eye that most of the retina would have been lit by the flash, and the light was never brighter than 5 e.f.c. This probably explains the differences between them and the records from the pigeon published by Kohlrausch & Brossa (1914) and by Graham, Kemp & Riggs (1935). In their experiments a bright light gave a prolonged negativity of the cornea after a very small positive wave, and their records with coloured lights do not show the double elevations which appear in Fig. 6. Restricting the field has sometimes changed the form of the response in the pigeon (though not in man nor in the monkey). With high intensities, the negative component (Granit's PIII, 1933) may well contribute to the shortening of the response, though it can scarcely account for the double elevations.

In the pigeon, therefore, the scotopic component is smaller than in man, but the two components are found, and the differences are what we might expect from an eye which is used almost entirely for diurnal vision.

Eyes with few cones

Cat and rabbit. The records in Fig. 9 are from the cat and make an interesting contrast to those in Figs. 1–8. The cat's eye has some cones, but as it is particularly adapted for night vision we should expect to find the scotopic component predominating. It can be seen that it does so, for there is no sign of the rapid component in the response to orange red or the shorter wavelengths, and with deep red there is no perceptible response. In the rabbit there is also a predominance of rods, and the records (Fig. 10) are like those from the cat. The chief difference is that the rabbit's eye is less sensitive and needs more dark-adaptation to give a large potential change.

The absence of any separation of the response into brief and slow components in the dark-adapted cat's eye is sufficiently explained by the predominance of rods. But we might expect that the two components could be distinguished if the rod contribution were greatly reduced by light-adaptation.

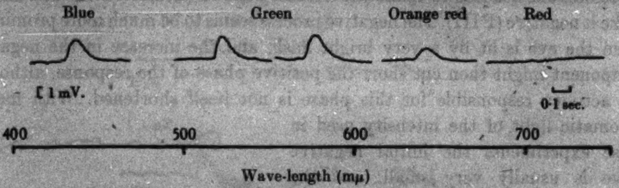

Fig. 9. Cat's eye. Response to flashes of different wave-length, brightness as in Fig. 1. Eye dark-adapted, pupil dilated with atropine. No sign of rapid component.

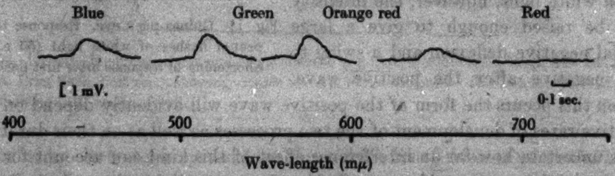

Fig. 10. Rabbit's eye. Response to flashes of different wave-length, brightness as in Fig. 1. Eye dark-adapted, pupil dilated. No sign of rapid component.

This does not happen. The effect of light-adaptation is to shorten the response to a very bright flash, but there is no stage in which we can make out (as in the monkey records in Fig. 3) a brief component not much reduced by light-adaptation and a slower component considerably reduced. In the rabbit there have been indications of this, but the discontinuity is far less than in eyes with a high proportion of cones.

The smooth contour and the progressive shortening of the response in the light-adapted eye is important, for it shows that we cannot regard the response curve as always made up of two components of fixed time relations but varying magnitude. In fact, if the slower part does represent the activity of a distinct mechanism, we must suppose that this activity can become shorter with light-adaptation and not merely smaller.

Guinea-pig. This conclusion is supported by records from the eye of the guinea-pig which has no cones, or only an extremely small number. The responses are like those in the cat and rabbit, and they, too, become much shorter when the light-adapted eye is exposed to bright flashes of white light (Fig. 11). Thus a receptor mechanism consisting almost entirely of rods can give a response to a flash which is just as brief as in a light-adapted eye with cones as well as rods. The simplest explanation of this is to suppose that the activity of the rod mechanism is curtailed when it is deprived of the sensitizing effect of the visual purple. But the analysis is complicated by the fact that in any case the electric response of the eye is to be regarded as the sum of two processes, one tending to make the cornea positive (Granit's P II) and one to make it negative (P III). The negative process seems to be much more prominent when the eye is lit by a very bright flash, and the increase in the negative component might then cut short the positive phase of the response, although the activity responsible for this phase is not itself shortened. With monochromatic light of the intensity used in these experiments the initial negative wave is usually very small, and the positive wave returns smoothly to the base-line without a swing to the negative. With white light, however, the intensity can be raised enough to give a large initial negative deflexion and a swing to the negative after the positive wave. When this occurs the form of the positive wave will evidently depend on the relative rates of development of the two processes as well as on their duration. It is uncertain how far an interference effect of this kind can account for the change in the response of eyes with few cones when light-adapted. An interference of positive and negative processes could not well account for duplex curves like those produced by orange-red light in man unless one or other of the processes were itself duplex, but it may come into play to some extent in all eyes when very bright flashes are used to produce a response in the fully light-adapted state. Whether it does so or not, however, it can be shown that there is an undoubted shortening of the retinal activity in these circumstances, since the optic nerve discharge is also definitely curtailed. Experiments dealing with this are described in the following section.

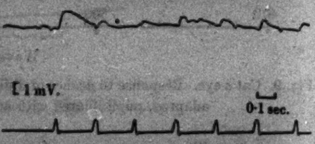

Fig. 11. Guinea-pig's eye. Response to repeated flashes of white light (55 e.f.c.). Shortening of response after first flash.

THE RESPONSE OF THE OPTIC NERVE

Judging by published records of discharges in single optic nerve fibres (Hartline, 1938; Granit, 1944) we could scarcely expect to find two distinct components in the response of the whole nerve, corresponding to rods and cones. But the eyeball response to a flash is altered so much by light-adaptation that we might expect to find a corresponding alteration of some kind in the optic nerve discharge. Actually the change is mainly one of duration, and the initial part of the discharge is relatively unaffected.

A detailed analysis of the different waves in the optic nerves of the cat and rabbit has already been made by Bartley & Bishop (1942). Their technique has been followed, and many of the present results are identical with theirs. The usual procedure has been to decerebrate the animal under ether and then to lift up one optic nerve stump by a silver hook which forms one electrode. The other electrode is fixed under the scalp to form an indifferent lead. With this arrangement there is little danger that the optic nerve records will be much distorted by electrotonic spread from the eyeball. Small doses of nembutal have sometimes been used to stop occasional reflex movements (e.g. flicking of the ears). Records were made from the eyeball in the usual way with a thread electrode on the cornea.

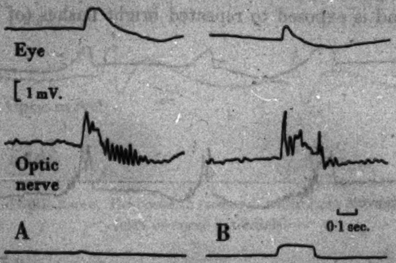

Fig. 12. Rabbit. Response of eye and of optic nerve to a flash of white light, brightness 102 e.f.c. A. Brief flash (0·05 sec.), showing double wave in nerve response. B. Flash lasting 0·22 sec., showing 'off' wave as well.

In the rabbit, as Bartley & Bishop have found, the intracranial part of the nerve dies from loss of its blood supply, and the electrode which supports it is leading from dead fibres. The responses are therefore entirely monophasic and are due to the arrival of impulses at the point where the nerve emerges into the cranial cavity. In the cat the responses are often di- or triphasic, possibly because some of the fibres survive better and give diphasic action potentials.

Typical records from the rabbit's optic nerve and from the corresponding eyeball are given in Fig. 12. In the optic nerve response to a brief flash of white light there are usually two main elevations, followed by a more gradual decline which may have small oscillations imposed on it. With a longer flash (Fig. 12B) there is also a large 'off' response when the illumination ceases.

Bartley & Bishop have worked out the conditions governing the appearance of the initial waves and have found that weak illumination gives only the second main wave, the first appearing in front of it when the light is stronger. At first sight it might be thought that these two waves are due to the rod and cone mechanisms respectively, but it is found that the second wave is not significantly reduced by light-adaptation, and that the two waves can also be recorded from the optic nerve of the guinea-pig (cf. Fig. 16). Thus a rod-and-cone explanation is not possible. The two waves may perhaps be due to direct and relayed transmission through the nervous pathways, but all that can be said is that they seem to be the consequence of the nervous organization of the retina and not of its having two kinds of receptor.

The effect of light-adaptation is shown in Fig. 13, where the eye is initially dark-adapted and is exposed to repeated bright flashes (of white light). The optic nerve response to the first flash subsides slowly after the initial waves, but the response to the later flashes subsides at once, the response of the eyeball showing a corresponding change.

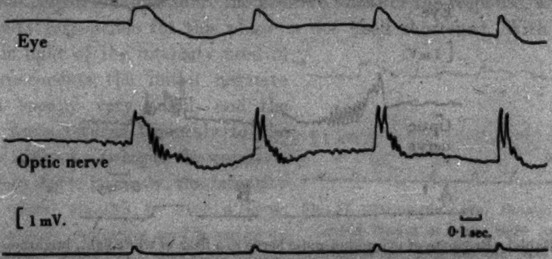

Fig. 13. Rabbit. Eye and optic nerve. Repeated flashes of white light (102 e.f.c.). Reduction in duration of nerve discharge after first flash.

Records from the cat's optic nerve are in general agreement with those from the rabbit. The eye is extremely sensitive, and the response to a brief flash usually continues for a second or more. The records are less easy to interpret as the responses are not strictly monophasic, but there is no doubt that the elevation which occurs after the initial wave (Fig. 14) is associated with a continued discharge of impulses, for these can be made audible by connecting the amplifier to a loud speaker. Fig. 15 illustrates the shortening of the discharge as the eye becomes light-adapted with repeated flashes. The records show even more clearly than in the rabbit how much the retinal response may be cut down without affecting the size of the initial waves in the nerve. It can also be seen that the second wave tends to become larger than the first as the flashes are repeated. The shortening of the discharge is not found only with repeated flashes; a period of continuous illumination has the same result. In both cases, recovery is rapid unless a very bright light has been used, a pause of 5–10 sec. in darkness producing a definite lengthening of the nerve discharge corresponding more or less with the increase in magnitude and duration of the retinal response.

Flashes of deep red (Wratten filter No. 70) have produced optic nerve discharges in the cat, though the retinal response has been too small to record accurately. The nerve discharge has been quite as long for a red flash as for a blue, and, in view of the great sensitivity of the eye, it is possible that the responses to red are mainly derived from the scotopic mechanism. Light-adaptation certainly reduces them as much as it reduces the response to shorter wave-lengths.

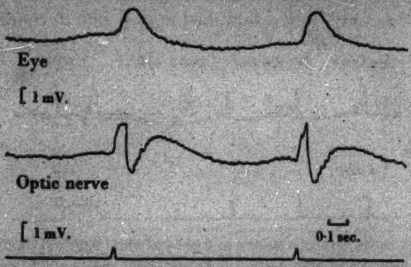

Fig. 14. Cat. Eye and optic nerve. Response to flashes of white light (0·1 e.f.c.) showing complex form of nerve potentials.

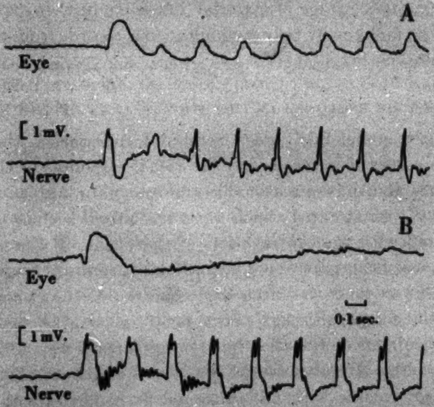

Fig. 15. Cat. Two records (different animals) of eye and optic nerve responses to flashes of white light (10 e.f.c. in A, 102 e.f.c. in B) at short intervals, showing reduction in duration of optic nerve discharge after first flash, with little reduction in initial waves.

It is less easy to obtain good records from the guinea-pig, for the decerebrate preparation is not so stable. Fig. 16 shows the preservation of the initial response in the light-adapted eye and the two waves which may be produced by the flash. As Granit (1944) has shown, there is very little 'off' discharge in the guinea-pig, but it is not completely absent (Fig. 16 B) and cannot therefore be regarded as due exclusively to cones rather than to rods.

The shorter duration of the discharge in response to a flash is therefore the chief difference which appears in these records of the optic nerve response when the eye is light-adapted. It is clear that a small and brief retinal effect is compatible with a large initial discharge of impulses, but there have been no obvious signs of a dual receptor mechanism. As the dual character of the retinal response has only been established in eyes rich in cones, attempts were made to record the optic nerve discharges in the monkey and pigeon. These have failed owing to shortage of material, but some further experiments have been made on the monkey by taking simultaneous records from the eyeball and from the occipital cortex.

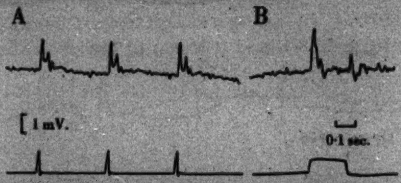

Fig. 16. Guinea-pig. Optic nerve discharges to flashes of white light (50 e.f.c.). A. Double wave in response to brief flash. B. Longer flash, showing 'off' wave.

THE ELECTRIC RESPONSE IN THE STRIATE AREA OF THE MONKEY

In the monkey, under fairly deep nembutal anaesthesia, the spontaneous activity of the cortex is reduced, and the visual responses of the striate area stand out clearly and show a reasonable uniformity for a given stimulus. But as the part of the striate area which is on the lateral surface of the brain is chiefly connected with the central parts of the retina, it was to be expected that the responses of this part would be due mainly to the photopic mechanism. This proved to be so, for it was often impossible to detect any cortical response from a flash of blue light, although a large potential could be recorded from the eyeball. With red light, on the other hand, large cortical responses were easy to obtain although the retinal potential was small and brief. This is shown in Fig. 17. The two records with an orange-red flash illustrate the effect of increasing the intensity of the light. A second cortical wave appears when the slower component of the retinal response is more obvious, but there is no reason to suppose that they are causally related, for repeated cortical waves have often been produced by deep red light giving only the initial retinal effect.

Evidently the mechanism responsible for the slower part of the retinal response has relatively little influence on the monkey's visual cortex, or rather on that part of it from which the lead is taken. This agrees with the view that the slower component is due to the scotopic mechanism. Possibly a different result would be obtained if the lead were on a part of the striate area connected with the periphery of the retina, but leads far back on the occipital lobe have not shown better responses with the shorter wave-lengths. A comparison of the responses on the lateral and mesial sides of the occipital lobe should give more evidence. It would be technically difficult, however, and has not been attempted.

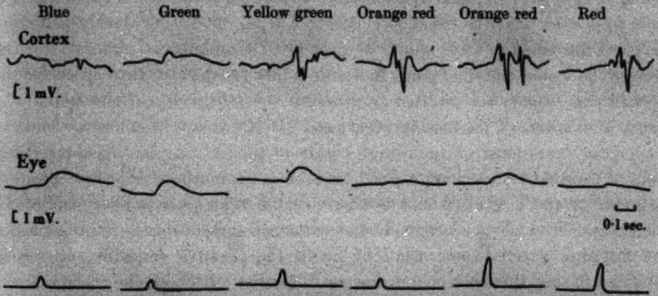

Fig. 17. Monkey. Response of eye and of visual area of cortex to flashes of different wave-length. The cortical effect is much greater with the longer wave-lengths. The two records with orange red show the effect of increasing the intensity (from 1·1 to 3·5 e.f.c.). At the lower intensity the dual response of the eye is just visible.

DISCUSSION

The records from the optic nerve and from the cortex do little more than emphasize the apparent lack of correspondence between the electrical response of the eyeball and the discharge of impulses from the retina. They can be explained on the hypothesis that the photopic mechanism is responsible only for the initial part of the eyeball response, but they cannot be said to give positive evidence of it. It is, indeed, unlikely that evidence of a dual receptor mechanism would appear in the optic nerve discharge even of the monkey or pigeon. It is presumably the function of the eye to furnish the brain with a coherent account of visual events, and, although it may employ two kinds of receptor, it has abundant synaptic connexions for welding their twin messages into one. This has been pointed out especially by Granit in his recent studies of single-fibre discharges.

As far as the eyeball response is concerned, the records from different animals certainly support the view arrived at from the human experiments.

The monkey and the pigeon both give the dual type of response with intermediate wave-lengths and only the photopic type with deep red; and in the cat, rabbit and guinea-pig the failure to detect a dual type of response agrees with what we know of the properties and structure of the eye. Records from the guinea-pig make it clear that the brief response of the light-adapted eye can occur in the absence of a cone mechanism, but this does not conflict with the hypothesis which contrasts the brief photopic response with the longer scotopic.

The investigation was planned in the hope that it would show what sort of information we might expect to obtain from the human electroretinogram, for it might have some clinical applications, and at least two other accounts of it have appeared recently (Spielberg, 1944; Karpe, 1945). Evidently a further study of the monkey's eye would be needed to complete the picture. For the present, however, the chief point to decide is how far the electric response of the eyeball can reasonably be used to measure the sensitivity of the retina, the range and speed of dark-adaptation, etc. If it cannot, the human electroretinogram loses most of its interest. Some of the limiting factors can be seen from the present experiments, and it is clear from them that the positive response of the eyeball is not a true measure of retinal activity under all conditions. With a very bright flash, for instance, the process responsible for the initial negative wave may cut down the positive response, and with repeated flashes this may be cut down still further, although the change in the optic nerve discharge is chiefly one of duration. It is only when the flash is brief, the light moderate and the field large that these additional complications of retinal activity are not obtrusive, and even then it is only with deep blue or deep red light that the response curve can be taken to measure pure scotopic or photopic activity. With these limitations, however, the response of the eyeball seems to be a reasonable guide to the performance of the receptor mechanisms. In animals, it is known to vary with the wave-length over a curve which coincides with that of the absorption of visual purple when the eye is dark-adapted and with the photopic luminosity curve when it is light-adapted (Graham & Riggs, 1935; Granit & Munsterhjelm, 1937; Granit & Wrede, 1937), and in man during the progress of dark-adaptation the response to a given flash increases along a curve which seems to run parallel to that of the increasing sensitivity of the retina (Adrian, 1945). The evidence is more complete for the scotopic than for the photopic type of response, but it is certainly enough to be encouraging.

CONCLUSIONS

1. Records of the electric response of the human eye to a flash of light have shown the presence of a rapid component apparently due to the photopic mechanism and a slower component due to the scotopic. Eyes rich in cones

(monkey and pigeon) are found to give similar responses. The two components can be most clearly distinguished in the response to a flash of orange-red light.

2. Eyes with few cones (cat and rabbit) give responses of the slower (scotopic) type in which it is impossible to distinguish two components.

3. In the eye of the guinea-pig, which has no cones, the response to a bright flash may be very brief when the eye is light-adapted. Thus a light-adapted rod mechanism can give a response which resembles the photopic effect.

4. The shortening of the response in the light-adapted eye may be due in part to an increase in the activity of the process which tends to make the cornea negative; but there is a definite shortening of the retinal activity, since the optic nerve discharge is curtailed.

5. Records of the discharge of the whole optic nerve in response to a flash have been made in the cat, rabbit and guinea-pig. It has not been possible to distinguish two components due to rod-and-cone mechanisms. The effect of light-adaptation is to shorten the discharge, but the initial waves caused by a bright flash remain large in spite of the great reduction in the electric response of the eyeball.

6. Records have been made from the striate area of the monkey's brain when flashes of different wave-length are thrown on the eye. The cortical effect is much larger when the photopic component is present in the electric response of the eye. Flashes of blue light which give only the slower component are often without effect on the cortical area which is connected with the central part of the retina.

7. The results support the hypothesis put forward to explain the dual electric response of the human eye and show the conditions which must be satisfied if the response is to be used as a measure of retinal activity.

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THE PRESENCE OF A SYMPATHOMIMETIC SUBSTANCE IN EXTRACTS OF MAMMALIAN HEART

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In a previous communication from this laboratory it has been shown that extracts of various mammalian organs, except placenta, contain a sympathomimetic substance, resembling adrenaline in its action on the blood pressure (Euler, 1945). The spleen was found to be especially rich in this substance, the extracts producing a pressor effect equivalent to about 10μg. adrenaline/g. fresh tissue. A closer study of the purified substance derived from spleen extracts revealed distinct differences between the active substance and adrenaline (Euler, 1946). The spleen extract, though about as active as adrenaline on heart and blood pressure, in amounts which give the same ferric chloride colour reaction, was distinctly less active on the isolated non-pregnant cat's uterus, the isolated intestine and in its pupil-dilating action. Furthermore, the active substance did not give the typical fluorescence reaction as described by Gaddum & Schild (1934) for adrenaline. Another difference was found in the action on the blood pressure after big doses of ergotamine or ergotoxine. Instead of giving the reversed action like adrenaline, the active substance still produced a rise in blood pressure, though the action was always substantially reduced. The actions of the substance were, in each type of test, roughly in agreement with those of the adrenaline-isomer *dl*-3 : 4-dihydroxy-*nor*-ephedrine, which, again, closely resembles *nor*-adrenaline or non-methylated adrenaline (Schaumann, 1931). It should be remembered that, as early as 1910, Barger & Dale postulated that the sympathetic effector substance would be different from adrenaline and more like a catechol compound with a non-methylated amino-group in the side chain. The results of Cannon & Rosenblueth (1937), concerning the release of sympathomimetic substances in vivo on nerve stimulation (sympathin), have strongly supported this view.

As to the occurrence of adrenaline-like compounds in the heart, Loewi (1936) has reported that extracts of mammalian hearts (rabbit) contain a small amount of a sympathomimetic substance giving the fluorescence reaction of adrenaline. Later Shaw (1938) found in the same organ small amounts of a substance giving the arseno-molybdate colour reaction equivalent to 0·015–0·04 μg. adrenaline/g. The specific increase in colour after addition of alkali was only moderate, however, indicating that the colour reaction could only partly be due to adrenaline. With a modification of Shaw's technique Raab (1943) found higher amounts of colour-giving substances in heart extracts from man and rat (0·7–1 μg./g.) though only a small fraction—as in Shaw's experiments—seemed to be adrenaline itself.

Using biological test methods, Cannon & Lissák (1939) observed that extracts of cat's heart contained some sympathomimetic compound in amounts corresponding to 0·5–0·8 μg. adrenaline per g. of fresh tissue as judged by the pressor action on the cat. The active substance was believed to be identical with adrenaline which was assumed to have been liberated from the adrenergic fibres of the organ. That such fibres may set free an adrenaline-like substance was originally shown by Gaddum & Khayyal (1936).

Quite recently, Hoffman, Hoffman, Middleton & Talesnik (1945) have reported that acetylcholine liberates an adrenaline-like body into the perfusion fluid of an isolated, atropinized heart. This observation is in accord with the results of McDowall (1944) who found that minute doses of acetylcholine stimulate the heart and sensitize it to adrenaline.

Summarizing the evidence, it may be stated that mammalian heart extracts contain an adrenaline-like substance in amounts up to 1 μg./g. Judging from Shaw's colour reaction only part of this is adrenaline itself.

By using the method of purification of organ extracts, described in earlier communications, it was hoped to be able to decide whether the active substance found in the heart conformed with adrenaline or whether it could be distinguished from it by biological or chemical methods.

METHODS

Extracts were made of heart muscle from cattle, horse and cat in the following way. The fresh organs were minced and extracted with 2 vol. of ethanol, to which was added 0·2 c.c. 10 N-sulphuric acid per kg. The filtrate was concentrated in vacuo to a small volume; the lipids being removed with ether. An extract of this type (crude extract) was used in the experiment illustrated in Fig. 1. This extract could be purified by extraction with ether containing some 5% brain-lipids or pure lecithine. The active substance taken up by the lipid-ether solution was again extracted by shaking with a small volume of 5% sodium sulphate. After precipitation of the sodium sulphate with 3 vol. of ethanol, filtering, and evaporation of the ethanol, the extract still contained a small amount of depressor material of choline ester type. This could be removed by treatment with fuller's earth in a congo-neutral solution.

The cats, used for the blood-pressure tests, were anaesthetized with chloralose and usually given 0·1 mg. ergotamine tartrate (Gynergen) per kg.i.v. and 8–10 mg. cocaine hydrochloride per kg.i.m. ½ hr. before the commencement of the testing in order to increase the pressor responses.

The crude extracts were used whenever the total pressor activity per g. of heart was determined. When the properties of the active substance were studied in greater detail, the purified extracts were used.

The blood-pressure actions were compared with *l*-adrenaline and *dl*-3:4-dihydroxy-*nor*-ephedrine.

Tests were also made on the isolated non-pregnant uterus of the cat. The usefulness of this test object, originally pointed out by Barger & Dale (1910), was borne out by our experiments. The fluorescence reaction was carried out according to the method described by Gaddum & Schild (1934).

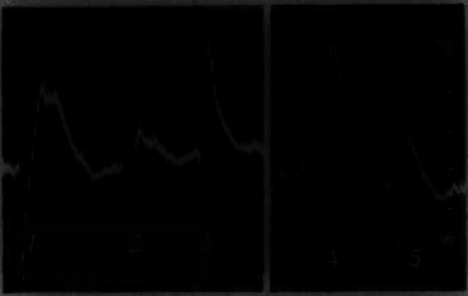

Fig. 1. Cat. Chloralose. Blood-pressure records. Crude extracts of cattle heart. (1) 0·3 g. (2) 0·06 g. (3) 1 μg. adrenaline. (Cocaine 10 mg./kg.) (4) 0·06 g. (effect equivalent to about 0·5 μg. adrenaline). (5) 0·06 g. extract treated with N/10-NaOH at 100° C. for 10 min. Time ½ min.

RESULTS

Blood pressure

The crude extracts of fresh heart muscle (whole heart) regularly caused a rise in blood pressure preceded by a short fall (Fig. 1). The effect in most experiments corresponded to some 5 μg. adrenaline per g. heart. When purified extracts were used, the depressor action almost completely disappeared and the pure pressor action was left (Figs. 2, 3). The blood-pressure response of the active extracts generally showed a typical difference from adrenaline, the initial rise in pressure with the former being definitely quicker. The active substance in this respect behaved more like *dl*-3 : 4-dihydroxy-*nor*-ephedrine (Fig. 3). After cocaine the effect of the heart extracts was increased in about the same proportion as adrenaline or *dl*-3 : 4-dihydroxy-*nor*-ephedrine. After doses of ergotamine or dihydro-ergotamine, sufficient to reverse the action of adrenaline (Rothlin, 1944), the pressor action of the heart extracts, like that of *dl*-3 : 4-dihydroxy-*nor*-ephedrine, was still present, though considerably decreased as shown in Fig. 3. The similarity between the two substances is

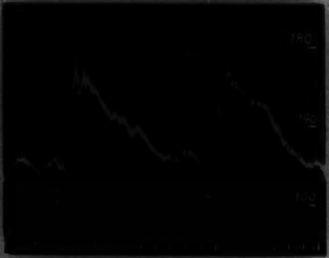

Fig. 2. Cat. Chloralose. Blood-pressure records. (1) 0·1 c.c. lipid-ether extract of cattle-heart extract. (2) 1 μg. adrenaline. Time ½ min.

quite obvious and suggests that adrenaline, if present at all, takes but an insignificant part in the action.

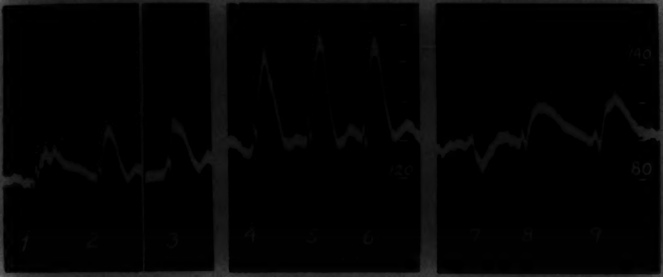

Fig. 3. Cat. Chloralose. Blood-pressure records. (1) 1 μg. adrenaline. (2) 1 μg. *dl*-3 : 4-dihydroxy-*nor*-ephedrine. (3) 0·1 c.c. lipid-ether extract of cattle heart, treated with fuller's earth. (Cocaine 8 mg./kg.) (4) 1 μg. adrenaline. (5) 1 μg. *dl*-3 : 4-dihydroxy-*nor*-ephedrine. (6) 0·1 c.c. lipid-ether extract of cattle heart, treated with Fuller's earth. (Dihydro-ergotamine 0·3 mg./kg.) (7) 3 μg. adrenaline. (8) 3 μg. *dl*-3 : 4-dihydroxy-*nor*-ephedrine. (9) 0·3 c.c. lipid-ether extract of cattle heart, treated with fuller's earth (same extract as in (3) and (6)). Time ½ min.

Isolated non-pregnant cat's uterus

Though the experiments on the cat's blood pressure after ergotamine in high doses or dihydro-ergotamine in doses of 0·2–0·3 mg./kg. seem to show conclusively that the active substance bears a much closer resemblance to catechol compounds without a methyl group linked to the amino-nitrogen than to adrenaline, it seemed desirable to test the action on the non-pregnant cat's uterus. As shown in Fig. 4, the heart extract had a much weaker inhibitory

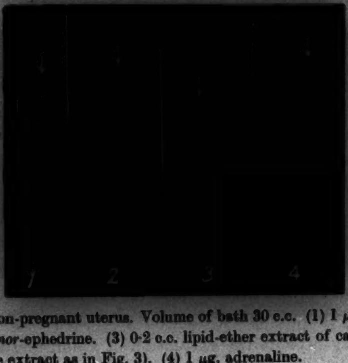

Fig. 4. Cat. Isolated non-pregnant uterus. Volume of bath 30 c.c. (1) 1 μg. adrenaline. (2) 1 μg. *dl*-3 : 4-dihydroxy-*nor*-ephedrine. (3) 0·2 c.c. lipid-ether extract of cattle heart, treated with fuller's earth (same extract as in Fig. 3). (4) 1 μg. adrenaline.

action than adrenaline, even in a dose which had twice the pressor action of the given amount of adrenaline. The heart extract thus compares much better with *dl*-3 : 4-dihydroxy-*nor*-ephedrine, the action of which is also shown in Fig. 4. Addition of a small amount of adrenaline to the heart extract, before adding it to the uterus bath, promptly caused an inhibitory action, demonstrating that any adrenaline which might have been present in the extract would have been effective.

Fluorescence and colour reactions

Purified extracts were matched with adrenaline solutions so as to give the same pressor actions. When tested for the fluorescence reaction, the adrenaline solution (10μg./c.c.) gave the characteristic strong fluorescence, whereas the heart extract did not give any definite reaction. The heart extracts showed a weak fluorescence before the addition of alkali, which disappeared when alkali was added. It may be concluded that the amount of adrenaline present in the extracts must have been small as compared with the other compound.

The colour reaction with phosphotungstic acid by the method of Folin, Cannon & Denis (1912) was found, in the purified extracts, to be of the same order as that in an equipressor solution of adrenaline. The same applied to the catechol reaction with ferric chloride. Both these methods, especially the latter, which was more reliable, could therefore be used with a reasonable degree of safety in order to evaluate the amount of active substance in the purified extracts.

DISCUSSION

The present results confirm the findings of previous investigators (Loewi, 1936; Cannon & Lissák, 1939) that heart extracts contain a substance in some ways resembling adrenaline. On the other hand, the results presented here do not support the opinion of these authors that the active substance is identical with adrenaline. Even if it be admitted that small amounts of adrenaline might have been present in the extracts, which appears probable since chromophile cell groups have been found around the heart, the present results clearly show that their significance must have been moderate. The biological effects as well as the chemical tests seem to leave no doubt that the substance responsible for the sympathomimetic actions in the heart extracts is different from adrenaline, and is, instead, intimately related to some substance resembling *nor*-adrenaline.

The criticism may be advanced that the purification method by means of lipid-ether selectively favours the extraction of a non-methylated compound. The fact that added adrenaline is extracted in about the same proportion as the active substance argues against this assumption. The comparatively large sympathomimetic activity actually found makes it improbable that greater amounts of adrenaline could be present in addition.

Cannon & Lissák suggested that the active substance was adrenaline because the extracts (1) strongly dilated the pupil, (2) inhibited the uterus, and (3) caused a fall of blood pressure after ergotoxine. It is probable that a quantitative analysis with purified extracts would have revealed certain differences from adrenaline. It should be borne in mind that the non-methylated compound of the type of *nor*-adrenaline is not entirely devoid of inhibitor action, although this effect is less prominent than its augmentor action. A reversed effect after ergotamine not infrequently occurred in our experiments, too, with less purified extracts, but this effect disappeared on further purification—notably after treatment with fuller's earth—without any significant loss of pressor activity before ergotamine. The reversed action is very likely due to the presence of vaso-dilator substances exerting their action more conspicuously after partial inhibition of the pressor action.

On the other hand, it appears that the activity found in our heart extracts agrees well with that of the sympathin (E) released on stimulation of the cardio-accelerator nerves in Cannon & Rosenblueth's experiments.

SUMMARY

1. Extracts of heart from cattle, horse, and cat contain a sympathomimetic substance resembling adrenaline in its action on heart and blood pressure. The active substance differs, however, from adrenaline in certain respects.

2. The action of the active substance is, like adrenaline, enhanced by cocaine, but is not as readily inhibited by ergotamine.

3. The inhibitory effect of the active substance on the non-pregnant uterus of the cat is much weaker than that of adrenaline in an equipressor dose.

4. The active substance gives the colour reactions with phosphotungstic acid and with ferric chloride about as strongly as adrenaline but not the fluorescence reaction.

5. The amount of the active substance corresponds to about 5 μg./g. of fresh tissue of adrenaline in its pressor action.

6. The similarities between the active substance and catechol-ethanol-amine on the one hand, and the postulated sympathin (E) on the other is noted, and its possible relation to these is discussed.

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THE DISTRIBUTION OF EXCITATION AND INHIBITION IN SINGLE-FIBRE RESPONSES FROM A POLARIZED RETINA

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The electrical theory of retinal excitation and inhibition by slow potentials has been based on a number of correlations between such potential changes in the retinal cells and characteristic excitatory and inhibitory effects reflected by corresponding changes in the impulse activity of the optic nerve. The arguments for the theory have recently been summarized in monographic form (Granit, 1946). Now, if currents, generated within the retina itself in response to illumination or sudden darkness, may excite or inhibit, so that the discharge of single optic nerve fibres can become blocked or accelerated at both 'on' and 'off', then how would such fibres respond to externally applied currents? Granit & Helme (1939) experimented along these lines with the frog's eye but did not succeed in recording from single fibres *during* polarization. The electrical field, established by the polarizing current, caused uncontrollably large oscillations of the base-line at the level of amplification necessary for single-fibre work. However, they managed to prove that the components of the electroretinogram and the discharge of the *whole* nerve could both be either increased or decreased by a change in the direction of a current traversing the retina.

In order to understand why it is necessary to isolate single fibres, let us consider in what manner individual optic nerve fibres might have their properties obscured by the behaviour of the whole nerve. Assume, for instance, that a certain percentage of the fibres were excited at onset and inhibited at cessation of polarization, and that another group of fibres behaved in an opposite manner; assume, further, that reversal of the direction of the current exactly reversed the properties of the two fibre types with respect to onset and cessation of polarization, then, as a rule, the effects in the two groups of fibres would cancel. Actually, when I ultimately succeeded in performing this experiment with single fibres, the earlier average results were found to have been obscured by just this source of error. The new experiments now to be described reveal details which it would have been impossible to discover with the aid of any less discriminative method.

TECHNIQUE AND PROCEDURE

As before, it proved impossible to record with micro-electrodes from a frog's eye during polarization. Nor was the experiment successful with a mammalian eye—in which single spikes are more easily obtained—if one polarizing electrode was inside and the other one outside the eye. Recording was prevented by the disturbed base-line. But it could be reasonably well performed with the cat's eye if the polarizing electrodes—well-chlorinated silver-silver chloride rods—were placed nasally and temporally on the outside of the eye as in Fig. 1. The animal was decerebrated, lens and cornea removed and the micro-electrode inserted, as illustrated by the figure. The polarizing device contained a commutator, a 50,000 Ω resistance in series with the eye and a galvanometer for measuring current strength. The impulses were led off across condensers to a balanced amplifier.

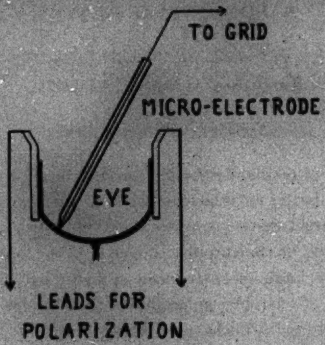

Fig. 1. See text.

RESULTS

General. Ten dark-adapted cats were used, and in twenty-five experiments on single fibres the polarizing currents elicited effects. Many more fibres were picked up, but it often happened that a successfully isolated fibre was outside the region in which the density of the currents sufficed to stimulate its receptor or synaptic connexions. It will be shown below that—at moderate current strengths—these structures and not the nerve fibre itself caused the effects obtained. Lack of an effect could not be remedied by increasing current strength indefinitely, since above 2 mA. base-line disturbances began to be troublesome. As a rule, however, fibres picked up on the inside in the neighbourhood of either outside polarizing electrode showed effects. Threshold effects were obtained with currents around 0·2 mA. The maximum strength used was 2·5 mA. Nearly all fibres studied were picked up at the nasal polarizing electrode.

The experiments were begun in dark-adaptation by investigating the effects of current strength, direction and duration. The fibre properties were next analysed by means of observations on the effects of variations in the intensity and duration of illumination. Polarization and illumination were then made to overlap, and finally a number of similar observations were repeated after light-adaptation.

Survey of fibre properties. All but one were of the on-off type so characteristic of the cat's optic nerve, i.e. they responded to both onset and cessation of illumination. Sometimes the off-effects were replaced by post-excitatory inhibition in the high-intensity range, sometimes they were maintained. (For a description of the properties of cat optic nerve fibres, see Granit (1945 *a*, *b*).) In order to be able to demonstrate inhibitory effects it is desirable to have spontaneously active fibres. The majority of fibres were of this type. In silent fibres it is often impossible to be certain as to whether absence of a response to polarization signifies inhibition or merely means lack of an effect.

In Fig. 2 are shown the typical antagonistic effects of polarization. The figure refers to a silent fibre. In record *a* the fibre responds to onset of polarization. The current was then reversed, and it is seen that in record *b* the fibre

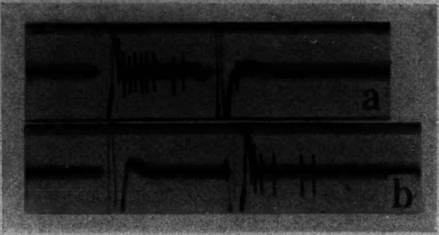

Fig. 2. Effects of polarization. Time in $\frac{1}{50}$ sec. in this and all records to follow. Current strength 1·0 mA. See text.

merely discharged when the polarizing current was broken. Thus on- or off-effects—to use the usual terms describing the responses to light and darkness instead of the terms make- and break-effects—were elicited according to the direction of the polarizing current. There was no absolute correlation with the polarity of the current in the sense that a micro-electrode in the nasal part of the retina would only pick up fibres responding with, say, on-effects when the nasal electrode served as cathode or off-effects with the same electrodes as anode. A couple of millimetres from a fibre discharging merely at the make of the current could be found another one discharging merely at the break of the same current. Both, however, reversed their properties when the current was reversed. The fundamental result of this work is therefore contained in the general statement that opposite currents, irrespective of the sign of the external polarizing electrode, as a rule, have opposite effects at make and break. Does this also hold for inhibition?

Before answering this question let us first recall that it is known from the work with single-fibre preparations responding to illumination that the off-effect presupposes pre-excitatory inhibition or inhibition at 'on' and that post-excitatory inhibition, i.e. inhibition at 'off', is commonly seen in certain fibres. The simplest way of demonstrating either form of inhibition is by using a spontaneously active fibre. The spontaneous rhythm is then blocked by inhibition at 'on' or at 'off'. Often both types of inhibition are mixed in the same fibre of the cat's retina, in a manner depending upon the strength of the stimulating light. Very few fibres in this eye are pure in the sense that they would respond only at 'on' or at 'off' (see Granit, 1945*a*).

Fig. 3 illustrates a spontaneously active fibre in which the spontaneous discharge is inhibited in *a*, at the make of the current, and accelerated into a vigorous off-effect at the break. In *b* is shown the response to the reversed current. There is now an on-effect at the make: a long pause of post-excitatory inhibition follows at the break, after which the spontaneous activity is resumed. Calling excitation *plus* and inhibition *minus* we have thus a perfectly symmetrical series: current passed one way leads to make +, break −; the other way, to make −, break +. This suggests that inhibition was present in originally silent fibres too though not demonstrable in them. The work on illumination has led to the conclusion that off-effects always are a consequence of pre-excitatory inhibition (see Granit, 1946). It can thus be said that polarization has provided a sensitive test by means of which a hitherto unknown property of the retinal sensory mechanism, a kind of reciprocal relationship, has been revealed.

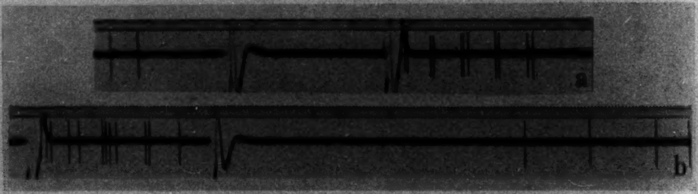

Fig. 3. Effects of polarization at 0·5 mA. See text.

The opposite effects of opposite polarizing currents with respect to on- and off-discharges have not always occurred throughout the whole range of current intensities (see below). In Table 1, however, all fibres that *within some range* of current strength have given symmetrical opposite effects have been marked by + and − as above. Uncertain effects have been marked by ±. Zero signifies that the fibre has been silent so that it has not been possible to demonstrate inhibition directly.

TABLE 1. Effects of polarization of eye

No. of fibres	Current one way ↑		Current reversed ↓	
	Make ('on')	Break ('off')	Make	Break
6	+	−	−	+
5	−	+	+	−
2	+	0	−	+
2	+	0	0	+
2	0	+	+	0
1	+	0	−	0
2	+	±	±	+
1	+	−	−	±
1	+	−	0	±
1	±	±	+	±
1	±	+	+	+
1	−	−	−	−
25				

The table shows that eleven fibres (6+5 in the two uppermost horizontal rows) have shown the symmetrical distribution of excitation and inhibition, illustrated in Fig. 3. Seven more fibres (2+2+2+1) have also responded with opposite effects to opposite polarizing currents, but in them the spontaneous activity has either been lacking or else so slow and irregular that it has been impossible to be certain of the inhibitory block. Of the seven remaining fibres four have shown an indication of responding to opposite currents with opposite effects, two (two last rows) have merely given excitation *or* inhibition, independently of the direction of the current.

Effect of current strength and duration. The fully symmetrical opposite effects of opposite polarizing currents were most noticeable at low and medium strengths, i.e. from threshold values to 1·5 mA. With stronger currents the inhibitory blocks were often broken up by excitation. In many fibres, however, symmetry persisted up to the maximum, 2·5 mA. The impulse frequencies of nearly all responses were measured and plotted as a function of current strength, but the variations noted are best illustrated by a few sample records. Cases in which the full symmetry was preserved and an increase of current strength merely led to an increase in frequency of the on- or off-spikes, elicited by opposite currents, have not been included. Figs. 4 and 5 are chosen to illustrate samples in which the response also changed type. In Fig. 4 the opposite effects are clearly visible at 0·5 mA. (records *a* and *b*). But, at 2·5 mA., inhibition at 'on' still prevails in *c*, corresponding to record *a* with respect to current direction, whereas in *d*, corresponding to record *b*, an off-effect has added itself to the on-effect. At the end of the latter are shown the typical base-line irregularities interfering with recording at this strength of stimulating current.

Fig. 5 also begins with two records, *a* and *b*, at 0·5 mA., illustrating opposite effects of opposite currents. With respect to direction of current, record *c*, at 1·5 mA., corresponds to *a*, and record *d*, at 2·0 mA., to *b*. Both on- and off-excitations are seen in the latter records. Thus, above a certain current strength, it may happen that the inhibitory effects are superseded by excitatory ones. Similar variations in response type are also seen with lights of various strength.

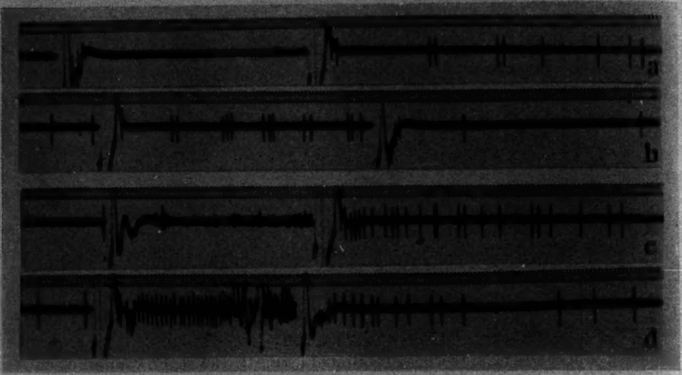

Fig. 4. Effects of polarization, fully explained in text.

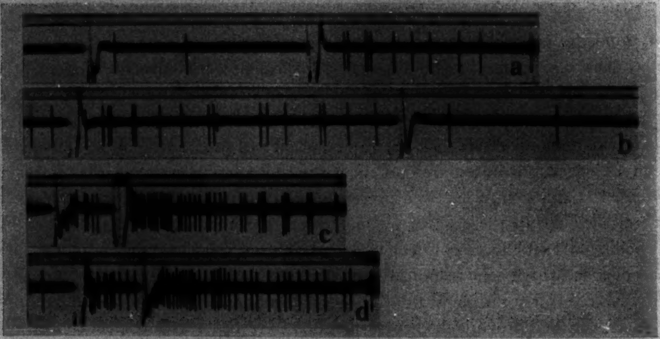

Fig. 5. Effects of polarization, fully explained in text.

The polarization device was operated manually and so did not permit shorter periods of stimulation than about 0·5 sec. From this duration to about 5 sec. there were very insignificant variations in the effects at 'off'. Sometimes the off-effect was slightly favoured, sometimes slightly diminished in frequency by an increase of stimulus duration. Mostly the off-response was independent of stimulus duration. Parallel experiments were made with light stimuli of different durations and a general similarity was noted.

It is well known that in most cold-blooded animals the average off-effect, within limits, increases with stimulus duration, and Hartline (1938) has drawn attention to the similar behaviour of off-discharges in single fibres of frogs. In cats, however, there have been no systematic observations on the effect of stimulus duration upon the off-discharges of single fibres. Within the limits used for durations of polarization, the observations here recorded, in which the duration of illumination was varied, show that in this animal the off-effect after illumination also is relatively insensitive to this particular variation, and that different fibres behave differently. On the whole it seems as if stability of type and reaction with respect to the intensity and duration of the stimulus were a feature of the frog's optic nerve fibre in contradistinction to the variability characterizing the fibres isolated in cats.

Interference between effects of illumination and polarization. If the light stimuli are too strong relative to overlapping polarizing currents or vice versa, then the stronger stimulus breaks through and elicits its own response. But by suitably adjusting the strength of the overlapping stimuli relative to one another interference effects can easily be demonstrated. When polarization series for different strengths of current were taken in dark- and light-adaptation it was often noted that the curves correlating impulse frequency and polarization strength (in mA.) were different in the two states of adaptation. Since the optic nerve may legitimately be held to be independent of state of adaptation, these results were taken to signify that the polarizing current had stimulated structures in the retina itself.

This conclusion received further support from the experiments in which stimulation by polarization overlapped with stimulation by light. In many of these experiments it was found possible to adjust the stimuli so that excitatory or inhibitory effects, caused by illumination, modified the effects of subsequent overlapping polarization. Thus, for instance, an excitatory effect, caused by polarization, was weakened if it fell into an inhibition that had been set up by light; or, again, if polarization caused excitation, this effect could be facilitated by a similar moderate effect of preceding illumination. Such experiments proved beyond doubt that—at least at moderate current strengths—in the first instance the retina itself and not the nerve fibres had been stimulated by the polarizing current (cf. also, Granit & Helme, 1939, for direct demonstration of effects of polarizing currents of the same order on the components of the electroretinogram). A priori it would also seem probable that the threshold of the retina to polarizing currents would be lower than that of the optic nerve fibres.

CONCLUSION AND DISCUSSION

If the general aspects of the electrical theory for generation of excitation and inhibition in the retina by its own component illumination potentials are isolated from specific assumptions, the remaining view is contained in the statement that opposite slow potentials, generated by illumination, have opposite effects. Component potentials of opposite sign have actually been demonstrated in the electroretinogram (see Summary, Granit, 1946), and it has been shown that pre-excitatory inhibition in many respects parallels the behaviour of the so-called negative component PIII, whereas the positive component PII coincides with excitation both at 'on' and 'off' (Granit & Therman, 1937). Later, post-excitatory inhibition was found (Granit, 1945*a*, *b*). This has hitherto been an isolated phenomenon, but the polarization test has now shown that pre- and post-excitatory inhibition in the same discharging structural element are connected in a way that throws new light on the functional organization of the retina.

In the assembly of fibres from a given region some respond to a current which traverses the bulb in a given direction with on +, off −, others with on −, off +. These effects are reversed with a reversal of current direction. Thus there will always in any given region be fibres responding to any given current with opposite effects at 'on' and 'off'. Either or both directions of current may be represented by component potentials of the electroretinogram. At the moment we need not consider all the consequences of both alternatives. Let it suffice to point out that there has turned up in these experiments a connexion between excitation and inhibition which for the sensory field could do what Sherrington's reciprocal innervation is doing for locomotion. In addition, there is also now a demonstration of a physical process that would be capable of accomplishing the desired result with regard to reciprocity.

This reciprocity mechanism for on- and off-effects must be a matter of internal organization of the retina, but one may well ask what purpose it would serve if it were activated by light stimuli. Sherrington (1906) himself raised and replied to this question when, long ago, as if in anticipation of the present results, he pointed out that visual contrast or induction provided a striking parallel to reciprocal innervation and that both in the retina and the spinal cord such mechanisms might be analogous with the 'make' and 'break' responses to galvanic stimulation of peripheral nerve. As to recent ideas for explaining contrast, etc., along the same lines, see Göthlin (1943) and Piéron (1943). The analogy seems more perfect than could ever have been anticipated before the actual discovery of the two forms of inhibition in the retina had been made. However, unless supplemented by the information provided by the delicate polarization test, the off-effects and the two forms of inhibition would have remained totally disconnected bricks in a puzzle game. Now one is beginning to see some physiological purpose in all these complexities. Further experiments along new lines with illumination will be necessary in order to complete the picture.

As to the comparison with peripheral nerve, recent results from this laboratory by Skoglund (1945) have shown that for nerve in catelectrotonus weak cathodal test stimulation leads to on (make) +, off (break) −, weak anodal test stimulation to on −, off +. The responses to both directions of test current are reversed for nerve in anelectrotonus. *Thus the retina behaves as if certain structures had some inherent differential polarity equivalent to that produced by the two types of electrotonus in peripheral nerve.* It is well known that there is a resting current across the intact bulb so that this idea is by no means inherently improbable.

SUMMARY

1. The eye bulb of the cat has been placed between two polarizing electrodes, and, with a micro-electrode, single-fibre discharges have been picked up from the optic nerve in response to polarization of the bulb with currents from 0·2 to 2·5 mA. Polarization has been shown to influence the retinal cells themselves. The effects, however, have always been picked up from the nerve.

2. Most fibres respond to the 'make' and 'break' of the polarizing current in an opposite manner: excitation at make, inhibition at break or vice versa. The fibres acting in this manner respond to a reversal of the current with a reversal of the effects at make and break.

3. Polarization is thus a sensitive test of properties inherent in the organization of the retinal cells. These properties suggest the presence of reciprocal innervation and thus throw new light on the general significance of the existence of pre- and post-excitatory inhibition in the retina.

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AN INVESTIGATION FOR THE PRESENCE OF A SKIN PROTEASE INHIBITORY FACTOR IN BURNED SKIN

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(*Received* 17 *January* 1946)

In former experiments (Beloff & Peters, 1945) it was shown that there is a gradual disappearance of the skin proteinase after burning at moderate temperatures, which is not due to local heat destruction. Two possible explanations for this phenomenon were advanced: one is that the enzyme leaves the skin, and the other that a factor which is inhibitory to the enzyme (an antiprotease) enters the damaged tissue. It was an attempt to settle this question which gave rise to the work described below.

It is of interest in connexion with this work that recently Zamecnik, Stephenson & Cope (1945) have reported an investigation of the peptidase activity of lymph and serum after burns. They identified a peptidase, with an optimum pH of 7·4, in normal lymph from dogs' legs, and observed that after burns to the dogs' extremities the peptidase activity rose abruptly in the lymph draining the affected area. They also identified an enzyme with similar properties in serum, erythrocytes, dog muscle, skin, subcutaneous tissue and in the bleb fluid from human burns. In calf and rat they found that an increase in peptidase activity of serum consistently followed a burn. We had also noted the presence of a peptidase in our skin extracts, but found that it could be largely separated from the proteinase.

In order to decide upon the presence or otherwise of antiprotease in skin, we were obliged to know more of the properties of such a factor. In work reported elsewhere (Beloff, 1946), it has been shown that the antiprotease for the skin proteinase is not the same as antitrypsin; it is present in high concentration in serum, and is found in the most soluble fraction of the serum albumin (for literature see Beloff, 1946). Among several procedures tried for separating proteinase or inhibitor from the inactive complex, the most successful was a defined fractionation with ammonium sulphate; by the use of this, an active inhibitor could be separated from the complex. Extracts from burned rat skin have been treated by this method, in order to find out whether an active inhibitor could be separated from them.

EXPERIMENTAL

The rats were shaved and burned at 60–63° C. for 1 min. using the burning iron previously described (Leach, Peters & Rossiter, 1943) and killed 1 or 2 hr. after burning. Samples of 'burned' and 'control' skin were extracted. The total decrease in proteolytic activity due to burning is given in Table 1. The 'residual skin' and part of the extracts were tested for their proteolytic activity in Exps. 2, 3 and 4. 3 c.c. of the remainder of the extracts were fractionated with ammonium sulphate. The precipitation of the protein present in the extracts followed the method previously described for plasma, i.e. the globulin was precipitated in a 50% saturated solution, and the albumin in a saturated solution of the salt (Beloff, 1946). The latter fraction was tested for its inhibitory activity on the skin protease. This is referred to as the 'Inhibitor Preparation'. The detailed results are given in Table 1. In Exps. 1 and 2 the inhibitory action was tested on protease preparations from 'acetone-dried' skin and in Exps. 3 and 4 on the protease extracted from the 'control' skin of burned animals. If the decrease in proteolytic activity produced by burning was due to the entrance of the inhibitory factor into the damaged tissue, then the action of the albumin fraction prepared from the burned skin extract would decrease the proteolytic activity of the control skin in comparison with that of the burned skin.

TABLE 1. Attempted separation of a skin protease inhibitor in burned skin by ammonium sulphate precipitation

Exp. no.	Temp. of burns (° C.)	Killed after burning (hr.)	Enzyme preparation	Inhibitor preparation	Increase in amino-N (mg.)	% decrease in proteolytic activity due to burning	% decrease in proteolytic activity due to addition of inhibitor preparation
1	62	1	Ex.A.D.	Nil	0·188	(Not investigated)	
			Ex.A.D.	In.C.	0·184		
			Ex.A.D.	In.B.	0·172		
2	61	2	Ex.A.D.	Nil	0·360	56	—
			Ex.A.D.	In.C.	0·348	—	—
			Ex.A.D.	In.B.	0·340	—	—
3	61	2	Ex.C.	Nil	0·308	44	—
			Ex.C.	In.C.	0·300	—	—
			Ex.C.	In.B.	0·272	—	12
4	60–61	2	Ex.C.	Nil	0·348	50	—
			Ex.C.	In.C.	0·308	—	—
			Ex.C.	In.B.	0·324	—	—

Ex.A.D. = Extract of acetone-dried skin.
Ex.C. = Extract of control skin.
In.C. = Inhibitor preparation from control skin.
In.B. = Inhibitor preparation from burned skin.

These experiments gave no evidence in support of the theory that the decrease in the activity of the proteolytic system of the skin is produced by the entrance of an inhibitory factor from the plasma into the damaged tissue.

No appreciable inhibitory action was observed from the preparations from extracts of burned skin, although in each experiment the burn produced considerable haemorrhage in the skin.

The effect of oedema fluid on the skin protease activity

In the above experiments there was a considerable loss of the oedema fluid from the burned area during the process of removing the subcutaneous muscle and adipose tissue. In two experiments therefore the oedema fluid itself was tested for an inhibitory effect on the skin protease. The rats were burned at 62° C. for 1 min. and killed 2 hr. after burning. The subcutaneous tissue containing oedema fluid was dissected from the skin and extracted in 3 c.c. of phosphate buffer. The weight of tissue + oedema fluid was approximately 2 g. The inhibitory effect of this extract on 5 c.c. of an extract of 'acetone-dried' skin was tested. The results, given in Table 2, show that the oedema fluid is inhibitory, but produces considerably less change than that observed after burning at this temperature.

TABLE 2. The effect of oedema fluid on the skin protease

Exp. no.	Enzyme preparation	Increase in amino-N (mg.)	% inhibition
1	Control	0·284	20
	+	0·224	
2	Control	0·216	17
	+	0·180	

DISCUSSION

It seems clear that a method of separating the inhibitor, which is successful with solutions of proteinase inhibitor, produces practically no inhibitor from burned skin. (It may also be mentioned that we could not recover inhibitor by precipitation with trichloroacetic acid; but we can lay no stress on this as it was a variable technique in our hands.) No evidence for the presence of inhibitor can be obtained from the burned skin, and little from the oedema fluid. The small amounts presumably entering the burned patch were not detectable. From this we conclude that our previous conclusion that the proteinase leaves the burned area for the blood or lymph is substantially correct; it would be more satisfactory to obtain a further proof by learning how to reverse the inactivation of the proteinase; so far we have failed in this. Though the enzyme leaves the skin, there is no evidence that it is actually toxic in the circulation; we know of no intravascular clotting; there must also be ample antiproteinase to deal with escaping enzyme. As a final check, on two occasions we injected the dialysed enzyme from the whole skin of a rat subcutaneously into both flanks of a rat. No ill effects and no change of weight were observed.

SUMMARY

1. No protease inhibitor can be recovered from the burned skin of the rat.

2. The decrease in proteolytic activity of skin after burning is mainly due to escape of the enzyme into the circulation.

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CHANGES IN INTESTINAL TONE AND MOTILITY ASSOCIATED WITH NAUSEA AND VOMITING

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The general features of the behaviour of the upper parts of the alimentary canal during nausea and vomiting are well known; but the associated disturbances of tone and motility in the intestines have received comparatively little attention. The few recorded investigations do not present a consistent account of the changes which take place, or provide a satisfactory explanation of their cause. The observations and experiments reported here show that in unanaesthetized dogs the nausea and vomiting produced by minimal doses of apomorphine are associated with a definite sequence of changes of central origin in intestinal tone and motility.

METHODS

Healthy adult dogs of either sex were provided with one or two Thiry-Vella loops of upper jejunum by operation under ether anaesthesia with full asepsis. After selection and resection of the required portion of intestine, as close to the ligament of Treitz as convenient, continuity of the gut was restored by end-to-end anastomosis. When two loops were prepared, this was done on a single occasion. About 12 in. of gut was first isolated and then divided at the mid-point. The ends of the two loops so formed were exteriorized in the nipple line on each side of the incision, and the incision closed in four layers.

Records of tone and motility of the loops were taken with the aid of condom rubber balloons of ample size (their diameter when inflated under minimal pressure was about 4 cm.), which were connected by thin rubber tubing to water manometers. The balloons were inflated to a pressure of about 15 cm. water after insertion into the upper end of the loop. The length of each loop was ascertained by allowing the balloon to be expelled from its lower end and then measuring the length of tubing inside the intestine, and this information enabled each balloon to be 'tethered' at about the mid-point of the loop during recording, by means of the wire device depicted in Fig. 1. It makes no difference to the form of the record if the balloon is allowed to move along the intestine, except that there is a considerable rise in tone and the disappearance of contraction waves when it is about to be expelled. If for some reason expulsion is delayed, these changes of motility are prolonged and may be misinterpreted.

The dogs were accustomed to stand quietly in a Pavlov stand during the recording and to receive subcutaneous injections without disturbance.

Considerable freedom of movement—including the movements of retching and vomiting—was permissible without detriment to the record, provided that the dogs were prevented from licking the ends of the loops, and that the tubing was supported so that its weight, or the movements of the dog, did not pull upon the balloons. Such forms of mechanical stimulation of the loops produced a local inhibition of tone and motility which was transmitted to the other loop and presumably also to the rest of the small intestine, via the abdominal plexuses. This form of 'intestino-intestinal' inhibition has been studied previously (Youmans, Meek & Herrin, 1938).

Nausea and vomiting were produced by means of subcutaneous injections of apomorphine hydrochloride (B.P., U.S.P., British Drug Houses). This method was preferred to such alternatives as distension of the intestine or administration of copper sulphate, digitalis, emetine, carbaminoylcholine chloride, etc., which lack the specific central emetic action of small doses of apomorphine, and may themselves produce intestinal or general systemic effects which would complicate the interpretation of the results.

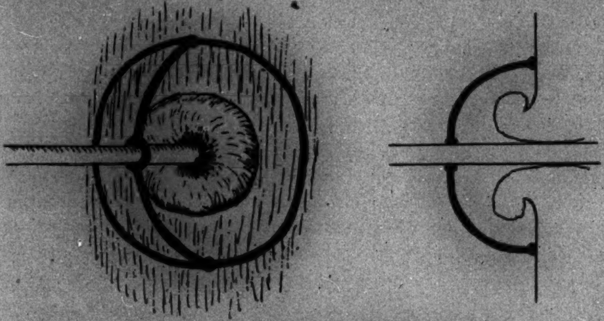

Fig. 1. The wire frame through which the balloon tubing is passed before entering the intestinal loop.

Dosage of apomorphine. It is well known that apomorphine has a depressant effect on the central nervous system of some animals, including the dog, and that samples may be contaminated with toxic impurities or decomposition products (Magnus, 1920). Care was therefore taken to employ the smallest dose which, in a single subcutaneous injection, produced from one to four episodes of nausea and vomiting during the first 30 min. This dose was first found by trial for each dog and then used each time; it varied from 0·02 to 0·10 mg./kg. body weight, and was freshly dissolved in 0·5 ml. of cold distilled water. Only solutions prepared immediately beforehand were used. With this dose, depression and muscular weakness were slight and transient. The dogs recovered quickly and showed a good appetite within an hour after injection.

The earlier observations were made on three dogs which had been provided for other purposes with a single Thiry or Thiry-Vella loop of the upper jejunal region. Four other dogs were each provided with two Thiry-Vella loops of the upper jejunal region. One of each pair was completely denervated at a subsequent operation by stripping from the vascular pedicle all visible nerves and non-vascular tissue for a length of 1–2 cm., a procedure which was followed in each case by the paralytic secretion first described by Claude Bernard (1859). Another noteworthy consequence of denervation was that evidence of visceral sensibility (discomfort on excessive distension or other mechanical stimulation of the loops) disappeared, although it remained in the untouched loop of the opposite side.

The dogs were used for recording about 7–18 hr. after the previous meal; and experiments were carried out in a well-warmed room. If the dogs had not eaten since the previous day, and the room was cold, shivering usually occurred and the records were found to be smaller in amplitude, and the intestinal tone lower, than under more comfortable conditions.

RESULTS

Nausea. After the injection of apomorphine, no effects whatever are apparent on the record of intestinal motility for a few minutes. The contractions continue with their accustomed regularity, at a constant level of tone, the rhythms in each loop being closely similar but not identical. General signs of nausea then appear; they vary a little in different animals, but although not very pronounced with the doses used in these experiments, they are fairly constant in form for a particular animal. The dog becomes subdued and still, yawns once or twice and licks its lips or swallows; salivation may be slightly increased. The respiratory movements are always affected and appear to be the best single indication of the onset of nausea; breathing becomes faster and shallower with the small doses of apomorphine used, though it is slowed and deepened with larger doses. The heart rate is slightly increased and dilatation of the pupils may be observed.

With the first signs of nausea, the tone and motility of both loops are quite abruptly inhibited, and the inhibition may be almost complete in about 30 sec. (Fig. 2). By this time, the tachypnoea is fully developed; and the intestine now shows an equally abrupt increase of tone, with some reappearance of contractions; a 'plateau' of apparently maximal contraction is usually reached and may be maintained for several seconds before a slow decline sets in and normal motility is resumed (Figs. 2, 3).

During this last phase (if retching does not supervene) the respiratory movements also resume their normal rate and depth.

Retching and vomiting. If the state of nausea, instead of subsiding, culminates in retching and vomiting, the rapid shallow breathing accompanying the rise of intestinal tone is seen to change into the slower and deeper respiratory movements of retching. This usually occurs some seconds after the 'plateau' of high intestinal tone has been reached, although where retching and vomiting are repeated in quick succession (Fig. 2) the interval may be short or almost absent. The retching movements, which end in a single powerful effort of emesis, produce synchronous disturbances in both records due to compression of the viscera. Between and after the episodes, intestinal tone and motility continue in apparently normal fashion.

The sequence of events just described has been reproduced with remarkable uniformity in a large number of instances of nausea, retching and emesis, without significant deviation in more than a small proportion of records. Occasionally vomiting is rapid and 'projectile' in character, with little or no premonitory nausea. In these cases the intestinal response may be partly or wholly lacking. The constancy of form and occurrence led at first to the tentative conclusion, subsequently disproved, that the changes were circulatory or mechanical in origin, rather than nervous.

In simultaneous records from the two loops, the observed effects are often closely similar, but never identical in extent or duration (Fig. 2); the only portion of the records in which synchronous excursions occur is during the efforts of retching and emesis. Sometimes the two records differ considerably in the details of the various phases, but the presence of these may almost always be clearly discerned. These observations, together with the fact that the whole picture of changes may be recorded when nausea alone occurs and when there is no sign of a retching movement, constitute good evidence against the mechanical origin of the changes. The possibility still remains that all the effects described have their origin in some interference with blood-flow through the loops produced by changes in respiration and circulation during nausea, whether or not this is followed by retching and vomiting; further evidence was therefore sought on this point by examining the behaviour of the loops in the two-loop animal after one loop had been denervated.

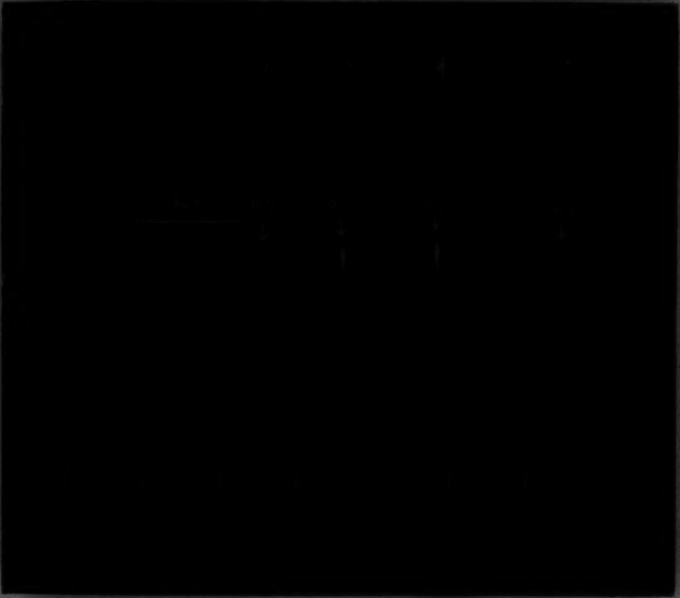

Fig. 2. Simultaneous records from a dog with two Thiry-Vella loops of jejunum. Apomorphine was given 3 min. before the first response shown. *N*, nausea (prolonged); *RV*, retching and vomiting. Time signal 60 sec.

Effect of denervation. Records were resumed about 7 days after the operation of denervation, by which time the motility and tone of the denervated loop had become apparently normal, while the behaviour of the untouched loop was exactly the same as before the second operation.

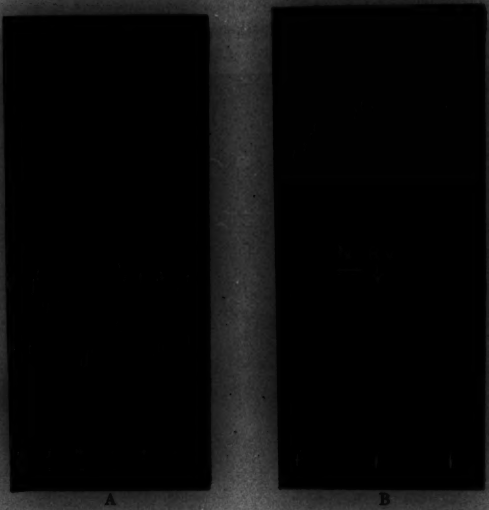

Fig. 3. Records taken from two different dogs, each provided with two Thiry-Vella jejunal loops, one of which had been denervated (lower tracing). Nausea and vomiting produced by apomorphine. A, nausea only; B, nausea (*N*) followed by retching and vomiting (*RV*). Time signal 30 sec. in A, 60 sec. in B.

The results were clear-cut; when nausea and retching occurred, the untouched loop behaved as before, but the completely denervated loop showed no significant changes (Fig. 3 A, B).

In one dog so treated, some doubt was entertained at the second operation as to the completeness of denervation of the pedicle; recording was begun a few days after operation, and the denervated loop was found to show some small and irregular changes in tone and motility, though not the well-defined response recorded at the same time from the other loop. After 7 days it was found that the 'denervated' loop now participated quite definitely in the characteristic changes associated with nausea; and after a further week the response was equally well defined in both loops.

This result, which occurred in no other animal, suggests that denervation had in fact been incomplete, though the remaining nerves were sufficiently interfered with by the stripping to prevent their influencing the loop for some days afterwards.

The results as a whole leave no reasonable doubt that the intestinal response described is due to a nervous discharge along the extrinsic nerves, associated with excitation of the 'vomiting centre' by minimal doses of apomorphine.

DISCUSSION

Babsky (1927) recorded by a balloon method the changes in intestinal activity following the administration of apomorphine to dogs provided with fistulae of the stomach, jejunum and ileum. Strong contractions, interpreted as antiperistaltic waves, were observed during nausea and vomiting. There are indications in the tracings, on which Babsky makes no comment, that these contractions were preceded by inhibition of the intestine. Oppenheimer & Mann (1941) studied the effects of nausea and vomiting produced by a variety of emetics on the activity of intestinal loops prepared in dogs by Biebl's (1930) method, which preserves intact the blood and nerve supply of the loop, and also the continuity of the intestine. They observed that nausea and vomiting were preceded by marked contractile activity; but owing to the limitations of the recording method necessary with this type of loop, the records give no indication as to whether relaxation of the intestine also occurred. Neither Babsky nor Oppenheimer & Mann provided proof of the origin of the changes observed.

Gruber & Brundage (1935), Slaughter & Gross (1938) and Gruber, Haury & Drake (1939) studied the effects of apomorphine on the tone and motility of the jejunum or ileum of dogs, using similar animal preparations and methods of recording to those used here. Their accounts show a certain measure of agreement with each other in that large and variable changes in tone and motility were observed, which often persisted long after the injection of the drug; the effects observed were assumed to be of peripheral origin, but no proof of this was offered. These findings are in notable contrast to the results of the present experiments, in which tone and motility remained apparently normal after injection of apomorphine, apart from the circumscribed responses associated with nausea and vomiting.

This discrepancy may arise from a difference in the route of administration of the drug. The primary object of the above experiments was the investigation of the action of apomorphine on the intestine, as compared with that of related compounds; and doses of apomorphine often greater than those given subcutaneously in the present work were administered *intravenously*, so that the time course of the concentration of apomorphine in the blood would probably be quite different in the two cases. There is experimental evidence (Hatcher, 1924), which this present work supports, that large doses of apomorphine are comparatively ineffective in producing emesis. For a short time after injection, nausea, retching and vomiting are almost continuous, but then there follows a prolonged period, characterized by depression, muscular weakness and nausea, during which movements of retching and vomiting are infrequent or absent altogether, and cannot readily be evoked by further injections. Further examination of this aspect of the action of apomorphine on the 'vomiting centre' seems very desirable.

Apomorphine has been regularly used in the present work because there is satisfactory evidence of its specific action on the 'vomiting centre' (Hatcher, 1924); but on several occasions the same intestinal response has been observed when nausea and vomiting occurred in relation to stimuli of other types. Distension of an intestinal loop, or the introduction of dilute acid, has sometimes been effective; and on one occasion the whole sequence of intestinal changes described here was observed when a greedy dog was given a large meal while recording was in progress; the animal ate it all very rapidly, and a few minutes later became nauseated and vomited.

The intestinal response described apparently has its origin in an excitation of central autonomic mechanisms, which may also account for several other accompaniments of nausea, namely, salivation, increased secretion of tracheal and bronchial glands, dilatation of the pupils, sweating, pallor, alterations in heart rate and blood pressure, changes in gastric tone and motility (Cannon, 1898; Barclay, 1936), antiperistaltic contractions of the duodenum (Gardiner, 1928; Ingelfinger & Moss, 1942), inhibition or stimulation of gastric secretion (Beamer, Friedman, Thomas & Rehfuss, 1944), and inhibition of pancreatic secretion (Bernard, 1856).

Experiments are in progress to ascertain the nervous pathways involved in the response.

SUMMARY

1. Intestinal tone and motility were recorded by a balloon and water-manometer method from Thiry-Vella loops of the upper jejunum in dogs, and nausea and vomiting produced by the subcutaneous injection of minimal doses of apomorphine.

2. Such doses of apomorphine have no detectable effect on the tone or motility, outside the periods of nausea and vomiting.

3. The first signs of nausea are accompanied by a rapid inhibition of intestinal tone and motility, followed by an equally rapid increase in tone, which may be maintained for several seconds before retching occurs; after this, normal tone and motility are resumed.

4. The response was abolished by denervation of the mesenteric pedicle to the loop; this and other arguments lead to the conclusion that the response is of nervous reflex origin and probably results from the excitation of central autonomic mechanisms.

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ON THE OCCURRENCE IN VITRO OF CELLS RESEMBLING OSTEOCLASTS

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During the early stages of the establishment of a 'pure' strain of endosteal fibroblasts in vitro, it was observed that a number of actively motile giant cells of unusual type were appearing fairly regularly among the cells growing out from explanted pieces of embryonic bone. As no description of similar cells in vitro could be found in the literature it was decided to investigate their properties, and, if possible, their source.

MATERIALS AND METHODS

The usual routine for hanging-drop tissue culture was employed; small fragments of membrane bone, obtained from the frontal bone of 13-day chick embryos, were explanted on to coverslips in a cock-plasma clot. After incubation for the requisite period, the cultures were fixed in Carnoy's fluid and stained subsequently either with carmalum, Meyer's haematoxylin and eosin, or Heidenhein's iron haematoxylin. The cultures were mounted in toto in balsam.

RESULTS

Examined after 24 hr. incubation, the cultures showed the usual outgrowth of an actively proliferating inner zone of spindle-shaped fibroblasts, and an outer zone comprising wandering cells which were both microphage and macrophage in type (Pl. 1, fig. 1). The former appeared to be heterophil granular leucocytes and were mostly degenerate. Of the latter, some may have come from spindle cells, which had wandered out into the clot; others were similar in appearance and staining reaction to blood monocytes.

In the living cultures, the cells shortly to be given the title of osteoclasts appeared in variable numbers, not as a 'strain' of cells deliberately and preferentially cultured, but as a comparatively small minority in a mixed cell population. They were found after 24 hr. incubation mostly in the outer zone among the wandering cells (Pl. 1, figs. 1, 2), but they did not always become free of the spindle-cell zone and often seemed to be enmeshed in the latter. They appeared before fixation as discrete bodies, characterized by a refractile, granular cytoplasm and standing out from the spindle cells as shining masses. Their shape was too variable for a mean type to be described, but two subdivisions were distinguished: one in which the cells, adhering to the underside of the coverslip, were flattened out by contact; the other, in which they were entirely surrounded by the medium, and therefore, if not spherical, at least 'three-dimensional' in appearance. The cells often occurred in groups of two or three. Their long axes were as often transverse as parallel to those of the spindle cells; it was not possible to follow their mode of progression, but it is hoped to do so with the aid of microcinephotography at a later date. The many nuclei could be seen in the living cell. The cell boundary was clear-cut and the outlines of the pleomorphic pseudopodia were fairly easy to follow. Many intracytoplasmic vacuoles were present in the larger cells (vide infra).

Observations on fixed and stained material, which it will be convenient to discuss under the following headings, confirmed and amplified the above findings.

Shape and size of cell

As measurement in one plane only was practicable, attempts to assess cell size accurately were abandoned. At one end of the scale were individuals little larger than a monocyte, containing one, two or more nuclei, and, at the other end, those of enormous proportions, with axes measuring up to 120μ and containing as many as 110 nuclei. The apparent size of a small spherical cell increases when it flattens itself against the coverslip, but 'flattens' is a relative term. In certain extreme cases the cell in contact with the glass was so attenuated that its nuclei became packed together in box-like form, the cell itself being invisible except through a $\frac{1}{12}$ in. objective (Pl. 1, fig. 3).

The cells varied greatly in size and functional activity; pseudopodia of varying appearance occurred, and some were occupied by one or more nuclei. Dumb-bell-shaped cells were seen, consisting of two multinucleate masses connected by a thin cytoplasmic strand. Whether they were in process of separating or fusing, or whether this appearance resulted merely from an exuberance of pseudopodial activity is impossible to say without the assistance of microcinephotography (Pl. 1, fig. 4).

Staining reaction

The outstanding characteristic observed was the presence of an inner, chromophil cytoplasmic zone merging into an outer, chromophobe zone. This was sometimes wavy and crenellated in outline. These zones may correspond respectively to an outer, tenuous and fluid exoplasm—the undulating membrane—and the inner, more substantial gelled layer of endoplasm. The exoplasm was itself sometimes ringed by a fringe of chromophil cytoplasm (Pl. 1, fig. 5).

The cytoplasm was strongly oxyphil and finely granular, but the fixing agent employed may have determined the granularity. The smaller types, containing one or two nuclei, often appeared more oxyphil than their larger relatives, probably because they were also frequently more spherical.

Multinuclearity

Regularity in the shape and size of the nuclei was a characteristic feature (Pl. 1, figs. 3, 5 and 6). The average nucleus was spherical or slightly ovoid, with a diameter of $3{\cdot}5\mu$. It was markedly basophilic. Rather reticular, it generally possessed one clear-cut nucleolus; more rarely, two were seen. The plastic nature of the nuclei was apparent in cells in which they were tightly packed. In the average cell, they were irregularly disposed, generally within the chromophil endoplasm. The average number per cell (estimated on a basis of approx. 1000 cells) was about six, with a highest recorded maximum of 110.

Many cells with similar staining reactions, and with well-marked exo- and endoplasm, were seen, little larger than a monocyte (and indeed frequently no larger) which contained one, two or three nuclei (Pl. 2, figs. 7, 8). These are regarded as immature individuals of the same series.

General observations

On no occasion was either mitotic or amitotic division seen, and, conversely, at no time were spindle cells seen fusing to produce fresh multinucleate masses. No fragments of bone matrix were observed within the cells or free within the medium, so that it is unlikely that the cells had transported any such into the clot. At times, however, refractile brownish crystals of undetermined origin, and at times erythrocytes, were seen within the cytoplasm.

After 48 hr. the cells were degenerate; the cytoplasm was coarsely reticular, intensely eosinophilic, and loaded with vacuoles. The nuclei were shrunken, of bizarre shape, and pyknotic. Soon after, little remained save an unrecognizable amorphous mass of granular oxyphil material.

Examination of cultures in the early stages of growth showed that the giant cells originated as such from the bony fragment, that they appeared well in advance of the outgrowth of spindle cells, and that they continued to wander out from the explant into the medium at various times after incubation began. The earliest giant cell seen emerged from the cut surface of the bone at the sixth hour, about three-quarters of its body being free within the medium. Within another 4 hr. it had wandered out, completely free. At the other extreme, cells could be seen (much more easily in the fixed than in the living culture) still very near the edge of the bone, as late as the 24th hour.

Having emerged, the cells wandered irregularly outwards from the implant. In their wake they left a track of digested or liquefied plasma (Pl. 2, figs. 9, 10). This point was demonstrated rather strikingly by direct fixation of specimens in Carnoy's fluid, without previously soaking in buffered saline to reduce the stainability of the plasma clot. This unusually vigorous liquefying property may have some functional significance.

It is, therefore, reasonable to assume that these cells are derived from bone and are osteoclasts. That they are not megakaryocytes is evident from the morphology of their nuclear substance. These cells are multinuclear; the megakaryocyte, polymorphonuclear.

DISCUSSION

In so far as no particulate matter resembling bone matrix was seen within the cells, nor free within the medium, the present experiments support the views of McLean & Bloom (1941) that osteoclasts have no osteolytic properties. On the other hand, the plasma-digesting property described does seem to point to possible proteolytic activity, and ingested intracytoplasmic collagenous material is notoriously difficult to demonstrate histologically (Vassos, 1940).

The origin of osteoclasts forms the subject-matter of a considerable literature. The views of the earlier workers have been summarized by Arey (1919), Ham (1932) and Bruno (1937). It may be said in general terms that some (Kolliker, 1873; Howell, 1890; Arey, 1919; Ham, 1932; Bloom, Bloom & McLean, 1941) regard the osteoblast as the source cell from which fresh osteoclasts derive; others, the marrow reticulum-cell directly (Jordan, 1921) or indirectly (Bloom, Bloom & McLean, 1941), whilst others (Mallory, 1911; Haythorn, 1929) favour the monocyte. It may be pointed out, however, that the close relationship between the marrow reticulum cell, the marrow endothelial cell and the monocytes tends to minimize the distinction between these views. The present observations support the last school. First, all stages were seen between cells resembling monocytes in appearance, and the fully developed osteoclast. Secondly, there is in the present series of experiments evidence of a functional nature. In the past, the derivation of osteoclasts seems to have been considered without realization of their vigorous motility; thus the only record of direct experimental work on this point which has been found is that of Kolliker (1873) who failed to observe motility in warm-stage preparations. It is difficult to believe that the mere coalescence of several relatively poorly motile individuals, such as reticulum cells, osteoblasts or osteocytes, could confer upon the resultant mass the property of great motility, which in the case of osteoclasts resembles that of monocytes rather than that of osteoblasts or other 'connective tissue' cells. Finally, if the tenuous exoplasm described above be accepted as the probable site of an 'undulating membrane'—a reasonable assumption—then, here, in the osteoclast, is another analogue of a monocytic characteristic.

The present experiments suggest that the cells arise by simple fusion and not by nuclear division, since no mitotic or amitotic division was observed in roughly a thousand nuclei scrutinized. They shed no light on the problem as to whether osteoclasts are directly responsible for bone resorption.

Although it would be unjustifiable to compare too closely conditions in vitro with those obtaining in vivo, the fact that all osteoclasts observed in vitro were grossly degenerate within 48 hr. indicates that their life should be measured in terms of hours rather than days. This is in accordance with the findings of Bloom *et al.* (1941). Working with material obtained from pigeons at different stages in the reproductive cycle, these authors describe periods of intense osteolysis which last for only a few hours and which are followed by periods of equally intense bone deposition. Their belief that in these circumstances osteoclasts are transformed into osteoblasts and marrow reticulum cells, and vice versa, is, however, not in accord with the present work.

No conclusions as to the fate of osteoclasts can be drawn from the present work. Although the description by Bloom *et al.* of a chromophil cytoplasmic zone round the osteoclast nuclei is confirmed, no support has been found for their suggestion that this zone separates into nucleated individuals. No budding off or splitting of exoplasm has been encountered, and their Pl. 2, fig. 2 can be interpreted in the light of the present experiments as showing merely an osteoclast in which the inner chromophil cytoplasmic zone is well demonstrated.

SUMMARY

1. In a proportion of cultures of embryonic chick bone, large multinucleated giant cells wander out from the explant.

2. These cells are considered to be osteoclasts and are derived from monocytes by fusion.

3. In vitro, they do not become transformed into other cell types.

It is regretted that precise magnifications cannot be given for the microphotographs which were taken in 1939. The optical bench and records were subsequently destroyed by enemy action and it has not been possible to estimate the bellows extension used. Acknowledgements are due to Mr R. Harrison for assistance with the microphotographs.

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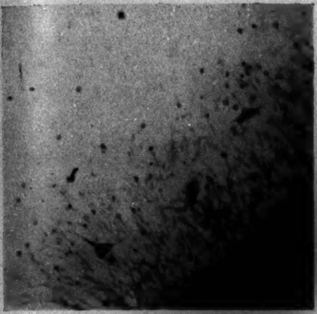

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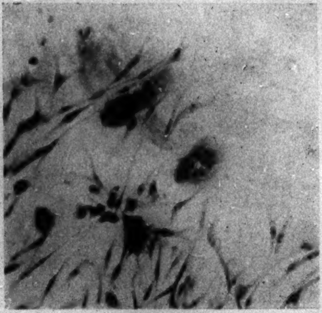

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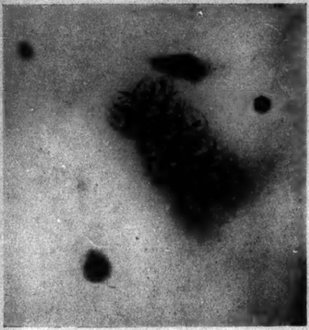

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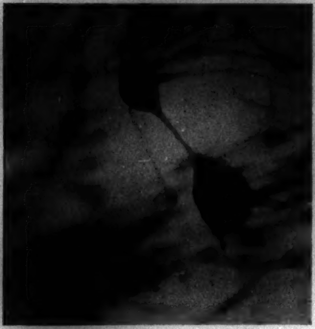

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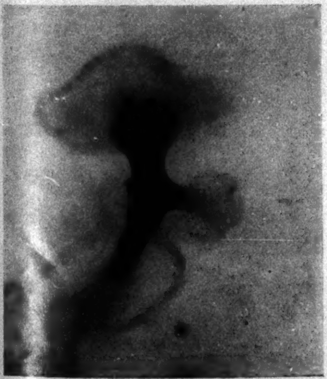

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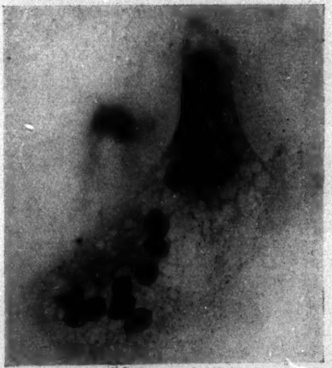

Fig. 6.

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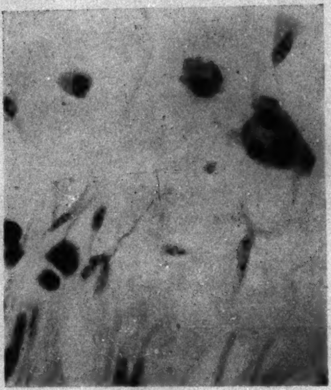

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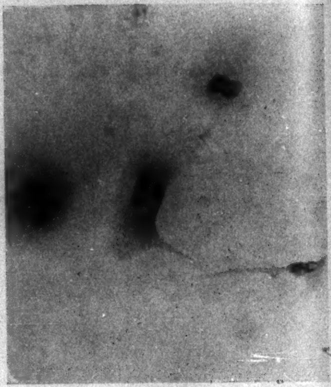

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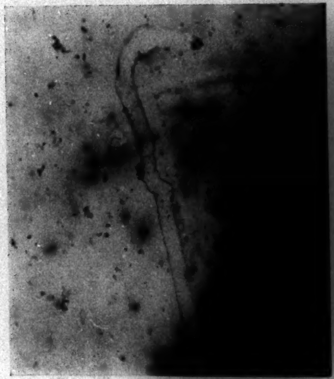

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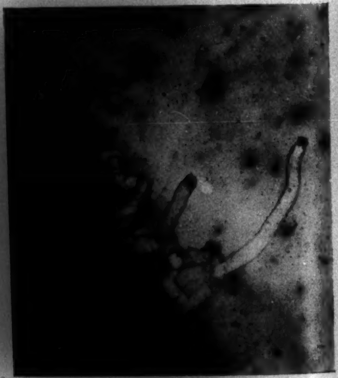

Fig. 10.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. 16 mm. objective, ×4 eyepiece. Edge of bone explant visible; three osteoclasts present in the spindle-cell outgrowth zone. 24 hr. culture.

Fig. 2. 8 mm. objective, ×2 eyepiece. Osteoclasts amidst spindle-cell zone. 24 hr. culture.

Fig. 3. 2 mm. objective, ×4 eyepiece. An osteoclast flattened against coverslip; nuclei pressed together in box-like fashion. 24 hr. culture.

Fig. 4. 4 mm, objective, ×4 eyepiece. An osteoclast in which two roughly spherical masses are connected by a thin cytoplasmic strand. 24 hr. culture.

Fig. 5. 3 mm. objective, ×4 eyepiece. The outer chromophobe and inner chromophil zones. 24 hr. culture.

Fig. 6. 2 mm. objective, ×4 eyepiece. Cytoplasmic vacuolation. 24 hr. culture.

PLATE 2

Fig. 7. 3 mm. objective, ×4 eyepiece. An osteoclast containing four nuclei. Several individuals with a single nucleus can be seen free and rounded within the clot. These are regarded as precursor cells. 24 hr. culture.

Fig. 8. 2 mm. objective, ×4 eyepiece. Osteoclasts containing two, three and four nuclei. 24 hr. culture.

Figs. 9, 10. 16 mm. objective, ×4 eyepiece. Tracts of liquefied plasma clot leading outward from the fragment of bone toward the periphery. Osteoclasts visible at the blind terminations.

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OBSERVATIONS ON PLASMA VOLUME ESTIMATIONS WITH THE DYE T 1824

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During work on battle casualties in the last 18 months of the campaign of the allied armies in Italy, it was particularly desired to relate the clinical states of casualties seen at various times after injury to their blood volumes at those times, and more than 200 blood-volume estimations were made with the dye T1824. At the times at which it was desired to know the blood volume, a proportion of these casualties had, in the preceding 5 hr., had doses of 16 mg. (gr. 1/4) and sometimes of 32 mg. of morphine, and some had received proprietary mixtures of papaverine and hyoscine. Bowler, Crooke & Morris (1944) have reported that if blood-volume determinations are made with the dye T1824 after patients have received injections of morphine or hyoscine or both, considerable fluctuations in the concentration of dye are found in a series of samples drawn over a period of several hours after the injection of dye. To estimate the plasma volume from the dye content of plasma samples, one may use either the dye content of a single sample of plasma drawn several minutes after the injection of dye, at a time at which it is assumed that the dye is properly mixed with the plasma and there has been negligible loss of dye from the circulating plasma; or an attempt may be made to correct for loss of dye by estimating the dye contents of a series of samples drawn at varying times after the injection of dye, and extrapolating through these values to zero time. The claims of Bowler *et al.* on the effects of morphine and hyoscine throw doubt on the validity of blood volumes estimated by the single sample method in patients who have received morphine or hyoscine, and from their published figures it can be seen that extrapolation through the values of dye concentration, obtained in their subjects who received morphine and hyoscine, may be impossible.

According to these authors fluctuations in dye disappearance curves are obtained if the dye is injected from 20 min. to 5 hr. after the injection of a mixture of 16·2 mg. morphine sulphate, 0·65 mg. hyoscine hydrobromide and 0·65 mg. atropine sulphate, or after the injection of morphine (dose not stated) or hyoscine hydrobromide (dose not stated), but not after atropine sulphate

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* Work undertaken on behalf of the Director-General of the Army Medical Services and the Medical Research Council.

(dose not stated).* Generally in subjects who had received morphine and hyoscine, dye concentration showed a preliminary fall, followed by a rise most marked 1–1½ hr. after the dye injection and in turn followed by a second fall; reference to their published figures shows that changes in dye concentration may be rapid and considerable as compared with the slow steady fall in plasma concentration seen in normal patients in the first few hours after they have received an injection of dye. If, however, morphine and hyoscine are injected after the injection of dye, even if only 10 min. after, normal dye disappearance curves are invariably obtained.

In our blood-volume estimations made on battle casualties at varying times after they had received morphine or hyoscine, however, very little evidence was found to support the claims of Bowler *et al.* For a variety of reasons it was often not feasible to withdraw samples from these casualties over a period longer than 40–50 min. after dye injection; for instance, prolongation of the period of blood-volume determination would have caused an unwarranted delay in transfusion or operation. It was therefore thought that the fluctuations in dye concentration described by Bowler *et al.* might have been missed in these blood-volume estimations because of too short a sampling period. The experiments now to be described were performed to test the effect of morphine and hyoscine on dye disappearances over longer periods after dye injection. The preparations of the drugs tested and the doses given are those that were used in the battle casualties seen with the allied armies in Italy. The morphine, hyoscine and atropine mixture studied by Bowler *et al.* was not given to any of the casualties seen, and its effects have therefore not been tested. Of chief interest has been the effect of morphine injections, since it is difficult to avoid the use of morphine for civilian or battle casualties, whereas hyoscine may be readily omitted from their treatment.

METHODS

Drugs and dosage used. All drugs were injected subcutaneously. The morphine used was a preparation supplied by the army, W.D. formula No. 5473, of morphine hydrochloride. Several different samples were used. Doses varied between 16 and 24 mg. (gr. 1/4–3/8). The preparations of papaverine and hyoscine given were either Allen and Hanbury's Alopon and Scopolamine or Roche's Omnopon and Scopolamine, and the doses given were 22 mg. (gr. 1/3) of Alopon or Omnopon and 0·43 mg. (gr. 1/150) of Scopolamine, the maximum doses of these drugs that casualties seen had received. The preparation of atropine used was a sample of atropine sulphate supplied by the army, and the dose 0·65 mg. (gr. 1/100). These drugs were from batches used in Italy during the spring of 1945 in the treatment of battle casualties, on whom many blood-volume estimations were at the time being made. After the administration of the drug in each case it was noted to be acting by its clinical effects, e.g. drowsiness and even deep sleep, loss of anxiety, pupil changes, dryness of the mouth, etc.

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* Since this paper went to press Dr Crooke has informed us that the doses of the separate injections of drugs referred to in the paper by Bowler *et al.* (1944) were usually morphine sulphate 16·2 mg., hyoscine hydrobromide 0·65 mg., and atropine sulphate 0·65 mg.

Estimation of dye. The methods employed in estimation of the dye content of plasma samples were two: (*a*) that described by Gibson & Evelyn (1938), and (*b*) the extraction method described by Crooke & Morris (1942). Further details of our use of these methods will be published later with observations on the effects of haemorrhage in battle casualties. The concentrations of dye in plasma after a single injection of dye were of the order of 1 mg./100 ml., and the dye was a sample of T1824 obtained from Imperial Chemical Industries. Estimations were made by a photoelectric photometer of the type described by Reeve (1944), in both methods with the red filters used by Gibson & Evelyn. Routine estimations of whole-blood haemoglobin, plasma haemoglobin, haematocrit and total protein were made on each blood sample to check the amounts of haemolysis and the constancy of the composition of the blood during the sampling. Since method (*a*) does not involve extraction of dye from the plasma nor dilution of extracted dye with the extracting reagent, and since, if the plasma is clear, more precise estimates of plasma-dye content are obtained than with method (*b*), it was preferred to method (*b*) unless the plasma samples were cloudy. In the experiments reported, with one exception, plasmas were clear, or not more than faintly cloudy, and the samples of a single plasma-volume determination were equally so as judged visually by the scattering of a beam of light passing obliquely through them.

RESULTS

Single plasma volume estimations after drug injection

In Fig. 1 are shown dye-disappearance curves on seven subjects at various times after they had received injections of morphine or papaverine and hyoscine, four convalescent volunteers, and three injured patients. In three

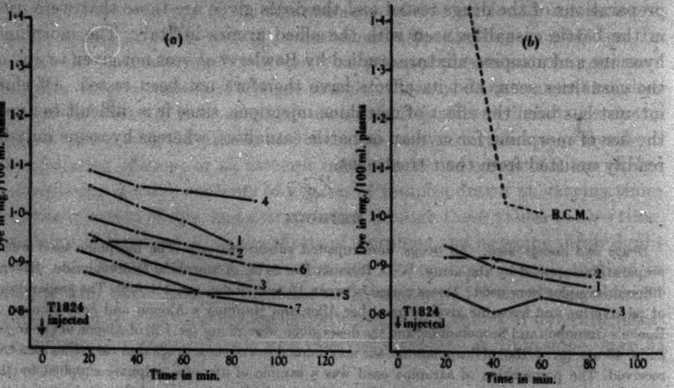

Fig. 1. (See opposite page)

of the volunteers estimations of dye were made by both methods. Details are given with the figure, and, in contrast, a dye-disappearance curve taken from the paper by Bowler *et al.* (1944) is also shown. It will be seen that there is no evidence here of abnormally rapid elimination or of a significant rise in dye concentration 60–90 min. after the injection of dye, as claimed by Bowler *et al.*

Double plasma-volume estimations separated by drug injection

Experiments similar to those reported by Bowler *et al.* were performed on seven volunteers. A preliminary plasma-volume estimation was first made, and, during the course of the determination of the rate of dye loss from plasma, an injection of morphine, or papaverine and hyoscine, or morphine and atropine was given. At periods varying between 30 and 65 min. after the injection of these drugs a second injection of T1824 was given, and the rate of disappearance of the dye from the plasma was again determined. The subjects on whom the experiments were performed were all soldiers of medical category A1 who had been warded for minor infections or injuries. They went without food for at least 16 hr. before the experiment and during the experiment, and were confined to bed for 12 hr. before and during the experiment. In each case the drug was observed clinically to be exercising a marked effect during the course of the second dye-disappearance curve. The results and details of the experiments are shown in Fig. 2*a*, *b*. Both methods of estimating the plasma content

Fig. 1*a*, *b*. Dye-concentration curves in four volunteers and three injured patients after injections of morphine, and papaverine and hyoscine. *a*, dye content of plasma estimated by Gibson & Evelyn's method; *b*, dye content of plasma estimated by Crooke & Morris's method.

Patient	Clinical notes	Drug injected	No. of min. before dye injection at which drugs were injected
1	Septic finger	Morphine HCl 24 mg. (gr. 3/8)	100
2	Appendicectomy 7 days before	Do.	100
3	Convalescent from mild enteritis	Do.	100
4	Small face laceration	Do.	100
5	Battle casualty. Lacerated scalp and minor lacerated wounds. Plasma vol. 1 hr. after operation, 14½ hr. after injury and after considerable transfusion chiefly before operation and drug injection. State of circulation good	Omnopon 22 mg. (gr. 1/3). Scopolamine 0·43 mg. (gr. 1/150)	180
6	Land-mine wound. Leg completely smashed. Great haemorrhage. Much transfusion. Amputation of leg through thigh. Plasma vol. 17 hr. after operation and 27 hr. after injury. State of circulation good	Do.	90
7	Battle casualty. Small wounds buttock, back and thighs. Perforating abdominal wounds causing two small perforations of jejunum. No peritonitis seen at operation. Transfused plasma before and very slow plasma transfusion during plasma-vol. estimation. No transfusion reaction during sampling. Plasma vol. 6 hr. after injury, before operation. State of circulation good	Morphine HCl 16·0 mg. (gr. 1/4) Morphine HCl 11 mg. (gr. 1/6) Evidence of being much under influence of morphine	330 200
B.C.M.	Fig. 1*b* from Bowler *et al.* (1944). 'Moderately shocked' with second and third degree burns of hands and face. (Some burned patients are known to lose dye more rapidly than normal patients but probably not at this rate)	Morphine sulphate (? dose)	90

of dye were used. It will be seen that here also, after the injection of these drugs, there is no evidence of abnormally rapid elimination of dye or of fluctuations in the dye concentration of the order described by Bowler *et al.*, though the same method of dye estimation has been used. In Fig. 2*b* the dotted lines B.C.M. show, in contrast, dye-concentration curves before and after the injection of morphine (dose not stated), taken from Bowler *et al.* (1944), when the dye concentrations were very similar to those here reported.

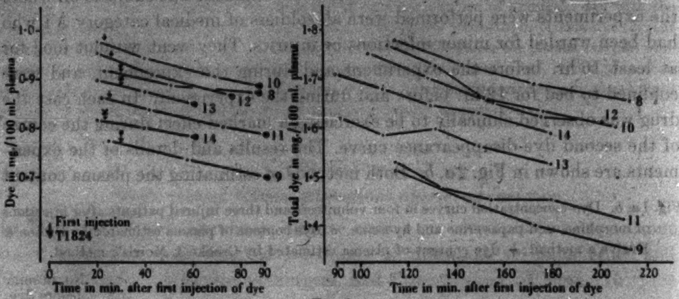

Fig. 2*a*.

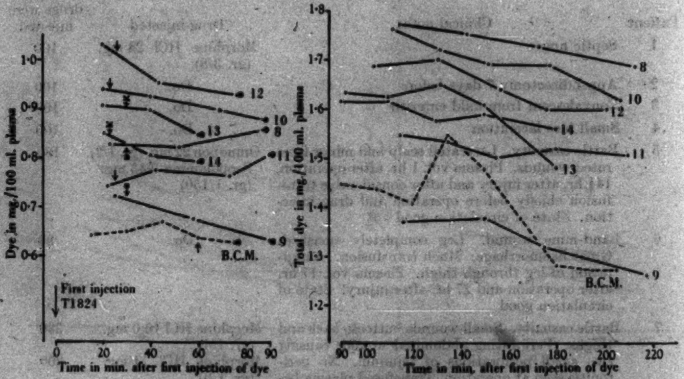

Fig. 2*b*. (See opposite page)

Comparisons of first and second plasma-volume estimates

Since the subjects, on whom the double plasma-volume estimations were made, were starved and kept in bed before and during the observations, it may be inferred that their plasma volumes remained fairly constant. If the drug injections do interfere with the second plasma-volume estimate, this estimate might be expected to differ appreciably from the first. The values obtained by both methods for first and second plasma-volume estimates by extrapolation through the first three points after dye injection in Fig. 2*a*, *b* are shown in Table 1 together, as an additional check, with the red cell volumes and total haemoglobin, calculated from the venous haematocrit, venous haemoglobin content and the plasma volume estimated by Gibson & Evelyn's method. With one exception, the second plasma volume on No. 13 attributable to cloudiness developing in the 'undyed' sample drawn before the second injection of dye, the variations observed were not greater than those expected from the various sources of error in the methods. This provides strong additional evidence against the interference of the drugs with plasma-volume estimates.

From Table 1 it can be seen that plasma-volume estimates made by the methods of Gibson & Evelyn and Crooke & Morris agree reasonably well.

Fig. 2*a*, *b*. Dye-concentration curves in seven patients, ages 21–36, convalescing from minor illnesses, before and after drug injection. First injection of dye given at 0. Second injection of dye given at •. Times of drug injection shown thus: ⬇ morphine and atropine; ↓ morphine only; ⤈ alopon and scopolamine. *a*, dye content of plasma estimated by Gibson & Evelyn's method. *b*, dye content of plasma estimated by Crooke & Morris's method.

Patient	Clinical notes	Drug injected	Time (min.) after injection of drug, when second injection of dye was given
8	Small 7-day-old haematoma of leg	Morphine 20 mg. (gr. 5/16). Atropine sulphate 0·65 mg. (gr. 1/100)	57
9	Convalescing from hand infection	Do.	60
10	Corneal ulcer	Morphine HCl 24 mg. (gr. 3/8)	65
11	Paronychia	Do.	65
12	Corneal ulcer	Do.	50
13	Conjunctivitis	Alopon (papaverine) 22 mg. (gr. 1/3). Scopolamine hydrobromide 0·43 mg. (gr. 1/150)	30
14	Corneal abrasion	Do.	37
B.C.M.	Fig. 3*a* taken from Bowler *et al.*	Morphine (? dose)	15

To avoid congestion in the figures a few of the curves have been slightly shifted.

In the second plasma-volume estimations total dye concentration is plotted against time. Since the two injections of dye were given close to each other, this gives a fairer measure of the rate of dye loss, since sampling errors cause variation in total dye concentration.

Relation of the double plasma-volume estimates to some observations of Cruickshank & Whitfield

These results in repeated plasma-volume estimations are of interest from a second viewpoint. Recently Cruickshank & Whitfield (1945) have claimed that after the injection of T1824 into cats an appreciable fraction of the dye was

TABLE 1. Plasma volumes estimated by the methods of Gibson & Evelyn and Crooke & Morris, before and after the injection of morphine, morphine and atropine, and papaverine and hyoscine. Total red cell and total haemoglobin values were calculated from the venous haematocrit, venous haemoglobin content and the plasma volume as determined by Gibson & Evelyn's method.

	Method of Gibson & Evelyn				Method of Crooke & Morris	
No. of subject	(a) Plasma vol. before drug (b) Plasma vol. after drug ml.	% difference between plasma vol. (b) and (a)	R.B.C. volume in ml.	Total haemoglobin in g.	(a) Plasma vol. before drug (b) Plasma vol. after drug ml.	% difference between plasma vols. estimated by Crooke & Morris's method and by Gibson & Evelyn's method
8	(a) 2740		2320	770	(a) 3020*	+12 (−5)*
	(b) 2690	− 2	2250	750	(b) 2710	+ 1
9	(a) 3200		3250	1035	(a) 3380	+ 5
	(b) 2950	− 8	2990	960	(b) 3120	+ 6
10	(a) 2680		2370	785	(a) 2740	+ 6
	(b) 2770	+ 3	2270	785	(b) 2670	+ 4
11	(a) 2980		2360	745	(a) 3200*	+ 7 (−5)*
	(b) 3150	+ 6	2380	760	(b) 3220	+ 2
12	(a) 2750		2670	860	(a) 2760	Nil
	(b) 2810	+ 2	2540	830	(b) 2800	Nil
13	(a) 3070		3200	1045	(a) 3060	Nil
	(b) 3460†	+13†	3460†	1140†	(b) 3350*	
14	(a) 3120		2820	935	(a) 3010	− 4
	(b) 3210	+ 3	2840	945	(b) 3210	Nil

* Extrapolation impossible through first three samples. Hence plasma volume calculated from *mean* of first three samples which should give plasma volumes about 5% too high.

† Sample before second injection of dye became cloudy on standing, resulting in overestimate of this plasma volume.

lost in the first few minutes, through absorption of the dye by some mechanism, probably the reticulo-endothelial system. This early and rapid loss was abolished either by a previous injection of carbon particles (India ink), or by a previous injection of dye. These authors suggested that with plasma-dye concentrations of about 1 mg./100 ml., because of this rapid dye removal, plasma-volume estimates may be too great by 20–30%, unless the absorbing mechanism is blocked by a previous injection of dye. In the repeated plasma-volume estimates here shown in Table 1 the dye concentrations were of the order of 1–2 mg./100 ml. plasma, and a second injection of dye was given between 60 and 90 min. after the first injection. From Cruickshank & Whitfield's work it may be assumed that the injection of dye given for the first plasma-volume estimate blocked the absorbing mechanism, and it would therefore be expected that the second plasma-volume estimate would be 20–30% lower than the first. This is plainly not so. Rather there is fairly close agreement between the two estimates. From Cruickshank & Whitfield's paper it would seem that they extrapolate through dye concentrations determined in the first 30 min. after dye injection, whereas the plasma volumes shown in

Table 1 were determined by extrapolating through three dye concentrations determined over a period of from 60 to 90 min. after dye injection, for both first and second plasma-volume determinations. It is highly improbable that this difference in the time of extrapolation would mask an absorbing mechanism of the activity described by Cruickshank & Whitfield. Additional evidence from the work of others may be quoted. Thus, Crooke & Morris (1942) quote two normal cases on each of whom three plasma-volume estimations were made at 80 min. intervals. In the first subject the second and third plasma-volume estimates were both greater than the first by 1%; in the second subject the second estimate was 6% greater than the first, and the third estimate 2% less than the first. The dye concentrations in the plasma were probably of the order of 1–4 mg./100 ml. Gibson & Evans (1937) also report repeated plasma-volume estimations on three subjects at 27 min. intervals, probably with dye concentrations of 0·5–1 mg./100 ml. plasma. In the first subject second and third estimates were less than the first by 5 and 3% respectively; in the second subject the second estimate was less than the first by 4%; and in the third subject the second estimate was greater than the first by 1%. These observations, all made on man, therefore indicate that the absorbing mechanism postulated by Cruickshank & Whitfield does not much, if at all, affect the constancy of repeated plasma-volume estimates made with T1824 at short intervals in man.

SUMMARY

1. The claim of Bowler, Crooke & Morris that anomalous plasma-dye concentration curves are found if the dye T1824 is injected in man, after the injection of morphine, has not been confirmed.

2. Plasma volumes determined with the dye T1824, before and after the injection of morphine and of proprietary preparations of papaverine and hyoscine, agree within the errors of the method, if the plasma-dye content is estimated by Gibson & Evelyn's method or by Crooke & Morris's method.

3. Evidence is advanced for man which conflicts with an absorbing mechanism, said to remove a considerable fraction of injected T1824 in the first few minutes after injection, and to be blocked by a previous injection of T1824 (Cruickshank & Whitfield). If two plasma-volume estimates are made within a short time on the same subject, the two estimates do not differ by more than the errors expected in the method.

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REFLEXES ELICITED BY VISCERAL STIMULATION IN THE ACUTE SPINAL ANIMAL

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In his description of receptor organs, Sherrington (1906) points out that the surface receptive field presents two divisions. The exteroceptive field, which is co-extensive with the so-called external surface of the animal, and the interoceptive field, its so-called internal surface which is usually alimentary in function. Little is known of the receptor organs of the latter surface. The afferent nerve fibres are, to quote Sherrington, comparatively few as judged by their number in the white rami. 'The poverty of afferent fibres from the interoceptive field is broadly indicated by the fact that we know no wholly afferent nerve trunk in the sympathetic system. The visceral constituent of the spinal ganglion is not present in all segments and is probably, even in those segments in which it is present, numerically the weakest of the three components.'

'The exteroceptive arcs', according to Sherrington, 'appear in most segments less closely connected with the visceral musculature than with skeletal musculature. The interoceptive arcs appear in most segments less closely connected with the skeletal musculature than with the visceral. In physiological parlance a resistance to conduction seems intercalated between the two. But both exteroceptive and interoceptive fields easily influence, through their nervous arcs, the musculature of the blood vascular organs.'

Mackenzie (1930) has described a viscero-motor reflex as one of the phenomena associated with visceral disease. This reflex is described as a contraction of skeletal muscle following visceral stimulation. Lewis (1942) points out that Mackenzie enlisted Sherrington's help, and the latter was able to produce movements of the abdominal wall by stimulating the bile duct or the central end of the superior mesenteric nerve. Subsequent work was done by Miller and his co-workers (1924, 1925*a*, *b*, *c*), who have investigated the reflex in decapitate cats. They found that squeezing the small intestine between finger and thumb, pulling the mesentery and faradizing mesenteric nerves produced contraction of leg and belly muscles with a rise of blood pressure. Lewis & Kellgren (1939) reported, however, that hard pinches of the intestine of decapitate cats did not affect skeletal muscle, whereas pinching the pancreas produced contraction of the abdominal muscles. In each instance a rise of blood pressure was recorded. Lewis & Kellgren also failed to elicit visceromotor reflexes from any of the solid abdominal viscera. They suggested that movements and pain which arise apparently from viscera are in fact the consequence of mesenteric stimulation. It will be remembered that Lennander (1902) had reached a similar conclusion as a result of surgical experience. He demonstrated that in man the solid organs and some of the hollow organs of the abdomen can be cut, burned, crushed or stretched without the subject being conscious of injury. Lennander concluded that the viscera were incapable of initiating pain, and that pain impulses originated only from the parietal peritoneum.

Much of the work on the sensory innervation of the intestine has been aimed at analysis of the mechanism of referred pain. Investigation of changes other than pain, produced by intestinal stimulation, has been neglected. Indeed, the inability of intestinal trauma to cause pain has led to a common belief that the viscera are insensitive to all forms of stimulation.

In this paper are recorded some of the results of intestinal stimulation as seen in the acute spinal animal. Preliminary observations showed that reflexes of intestinal origin could be most easily elicited when the spinal cord was transected in the upper thoracic region. Previous workers have used the decapitate animal, i.e. with cord transected in the upper cervical region. Miller and his colleagues, and Lewis & Kellgren removed the head, as described by Sherrington (1909). McDowall (1942) prefers to turn a decerebrated animal into a spinal animal by occluding the carotid and vertebral arteries. In the majority of cats this results in 15 min. in a spinal preparation in which leg movements result from stretching the intestine, but cutting has no effect. McDowall warns against over-ventilating the preparation, a warning given also by Lewis & Kellgren.

In the following account the reflex activity of three preparations is compared: (1) decerebrate animal with cord transected in upper thoracic region, (2) decapitate animal, (3) decerebrate animal.

METHODS

Cats were used in all experiments, the preliminary transection of the brain stem being made under deep ether anaesthesia. Decerebration was done through a hole in the cranium, the brain being removed anterior to the tentorium cerebelli; bleeding from the brain stump was controlled by tying the common carotid arteries in the neck and applying digital pressure to the vertebral arteries. The spinal cord was cut after removing the lamina of the overlying vertebra; the dura mater was opened and the cord cut across with scissors. For decapitation the carotid arteries were tied in the neck and the spinal cord was exposed by removing the lamina of the axis. The cord was cut intradurally, and the cranial cavity was pithed with a probe passed upwards through the foramen magnum. Positive-pressure ventilation through the tracheal cannula was begun at the time of pithing. All animals were left for 3 hr. to excrete the ether.

The abdomen was opened by a diagonal incision in the abdominal wall, running forwards and downwards from the renal area towards the mid-line and exposing the parietal peritoneum. Blood was not allowed to enter the peritoneal cavity, care being taken that all bleeding had ceased before the peritoneum was cut with scissors. It was possible, through a 2 in. incision, to produce any part of the intestine and the organs of the same side. To keep the environment of the exposed viscera as constant as possible, the trunk of the animal was submerged in a deep bath of Locke's Ringer solution (Bain, 1938) maintained at 38–39° C.

Movements of the hindlimbs were recorded by attaching the foot directly to an isometric myograph with a weak spring, by means of a wire stirrup strapped to the foot with adhesive tape. In other experiments the movements of a single muscle, e.g. gastrocnemius, was recorded isometrically in the usual way. In the majority of experiments the movements were observed by eye. Blood pressure was recorded with a mercury manometer from the central end of a divided carotid artery, using saturated sodium bicarbonate solution as an anticoagulant in the recording system.

RESULTS

Decerebration with chordotomy. In these experiments the spinal cord was divided between the eighth cervical and first thoracic roots, and the animal was decerebrated immediately afterwards.

This preparation had the characters of a decerebrate animal in the head and forequarters but of a spinal animal in the trunk and hindquarters. Breathing was maintained by the diaphragm. The hindlegs had good tone, and the muscle jerks were strong and sensitive. Handling or squeezing the paws caused flexion with crossed extension, the movement usually being a rhythmic stepping or a shaking-off. Tickling the hairs of the scrotum or labia produced flexion of the hind limbs, often with a superposed rhythmic walking movement. Gently squeezing the parts sometimes produced flexion, but usually strong extension, of the legs, with superposed stepping movements. The muscles of the abdominal wall were very sensitive to stretching, a gentle tap on the surface producing a sharp twitch of the underlying muscles.

Stimulation of the abdominal viscera caused movement of the hindlimbs, contraction of the belly muscles and rise of carotid blood pressure (Fig. 1). These responses followed, for example, pinching the small or large intestine with forceps, or gently squeezing a short length of bowel between finger and thumb. The responses of the limbs had many patterns. Some animals abducted the limbs and flexed them on the belly, others extended the limbs strongly downwards and backwards, while some first flexed the legs and then extended them. Most commonly initial extension was followed by, and merged into, rhythmic alternating flexion and extension which resembled walking or trotting. It was noted that the pattern of response to squeezing the external genitalia usually forecast the pattern of response to visceral stimulation, if the latter elicited movements.

The visceral stimulation produced also a rise of blood pressure. The rise was usually small, of the order of 20–40 mm., but rises of 80 mm. have been recorded (Fig. 2). A dissociation between the leg and vascular responses was sometimes seen, when animals which had lost the leg response still gave good blood-pressure responses. This disappearance of certain of the reflexes will be referred to again later.

Decapitation. These animals were prepared by pithing the cranium in the way described previously. They were kept alive by positive-pressure ventilation of the lungs for the rest of the experiment. During the subsequent 3 hr. these preparations showed progressively increasing activity of the limb. The muscles had good tone, and the muscle jerks were brisk and sensitive in all limbs. In the hindlimbs the flexion and crossed extension reflexes were strong and sensitive. Flexion reflexes were present in the forelimbs but were less strong and less sensitive. A scratch reflex was easily elicited from a large saddle-shaped area of the back of the neck and trunk.

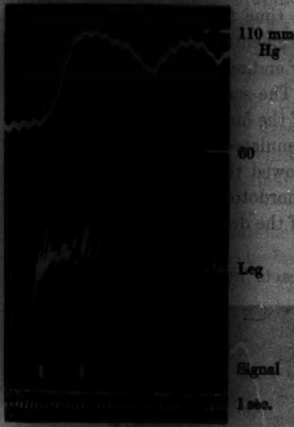

Fig. 1.

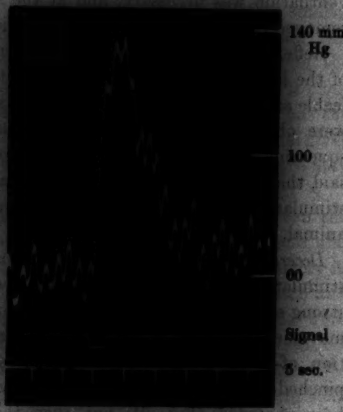

Fig. 2.

Fig. 1. Record of leg movements and blood-pressure changes on pinching the jejunum. Decerebrate cat with spinal cord transected between C_8 and T_1 roots. B.P. from carotid artery. Movements of left leg recorded by attaching foot to isometric lever.

Fig. 2. Rise of carotid blood pressure on pinching ileum. Decerebrate cat, spinal cord cut between C_8 and T_1 roots.

The liveliness of the reflexes elicited from the skin and of the muscle jerks contrasts with the weak leg movements which followed visceral stimulation. Stimulating the small or large intestine produced no, or but weak, movements of the hindlimbs. To produce these weak responses it was necessary to stimulate the bowel by drastic means, e.g. by pinching with forceps sufficiently hard to rupture the muscle, or by squeezing 1 or 2 in. of the bowel hard between finger and thumb. Movements could be easily elicited, however, by stimulating other organs. Pinching a small piece of the pancreas, squeezing an ovary, and pulling the intestinal mesentery were effective. Stimulating the intestine caused a rise of blood pressure, of the order of 30 mm., this rise of pressure occurring even if there were no limb movements.

The difference in reactivity between the decapitated and the decerebrated-chordotomized preparation suggested an investigation of a decapitated animal with chordotomy. Decapitation, after section of the spinal cord between the eighth cervical and first thoracic roots, gave a preparation in which artificial ventilation was necessary, and at the same time the lower spinal cord was removed from the influence of the cervical segments.

Reflexes elicited by stimulation of sensory endings in the skin and muscles of the hindlimbs were strong and sensitive. The scratch reflex was absent or feeble and slow to appear. Good responses of the hindlegs and abdominal wall were obtained when the intestine was stimulated by pinching or gently squeezing. Rises of blood pressure also followed the stimulation. It can be said that the reactivity of the decapitate-chordotomized animals to visceral stimulation may be equal to the reactivity of the decerebrated-chordotomized animal.

Decerebration. The decerebrate animal reacts little, if at all, to visceral stimulation. Decerebrate cats which had strong extensor hypertonus did not show movement of the limbs or palpable alteration of tonus when the intestine was pinched. Rises of blood pressure have been recorded (Fig. 3), but even this response was absent in some preparations. The insensitivity was not confined to the small and large intestine; stimulating the pancreas and mesentery was also ineffective.

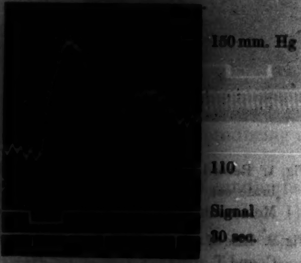

Fig. 3. Rise of carotid blood pressure on pinching jejunum of decerebrate cat.

The absence of response was not due to a failure to stimulate visceral nerves. Pinching the duodenum and pancreas produced contraction of the panniculus carnosus muscle, as described by Ashkenaz (1937), demonstrating that impulses were entering the spinal cord.

In the decerebrate animal the legs are held rigid by the muscular hypertonus, and it may be that the absence of limb movements is due to a splinting action of the contracted muscles. This would not account, however, for the absence of vascular responses in these preparations. That some other mechanism is involved is suggested also by the behaviour of a cat, with the brain stem cut between the superior and inferior colliculi, in which the limbs had low tone and the flexor responses of the hindlegs and the scratch reflex were present; even in this animal, visceral stimulation had no effect. In this experiment it was clear that the limbs could move as part of a reflex from the skin, but not from the viscera.

Comparison of the preparations. The decerebrated-chordotomized animal is a convenient and sensitive preparation for the examination of the reflexes associated with visceral afferent impulses. The viscero-motor and viscero-vascular reflexes are readily elicited by stimulating the intestine and other viscera. Generally the decapitate animal is not as sensitive to intestinal stimulation as the former preparation. Not only does stimulation produce smaller movements of the legs, but stronger stimuli are required to produce them; this animal is, however, sensitive to mesenteric stimulation and pinching the pancreas. On the other hand, the decapitated-chordotomized animal is comparable in sensitivity to the decerebrated-chordotomized animal, but has the disadvantage that artificial ventilation is needed. The decerebrate animal gives no limb movements on intestinal stimulation, and blood-pressure changes may be absent.

In all the spinal preparations there was a striking difference between the state of the reflexes elicited by stimulation of the skin and skeletal muscle, and those elicited from the viscera. The former showed a high degree of recovery in the 3 hr. elapsing after chordotomy. The flexor, crossed extensor, stretch and other reflex responses obtained on stimulating the external genitalia were strong and sensitive. In contrast, animals with such lively somatic reflexes might have no, or but poor, viscero-motor reflexes. This was the usual condition of the decapitated animal, but might obtain in the other preparations also. It must be emphasized that the state of the somatic reflex arcs is not an index of the state of the visceral arcs. An animal which gives good demonstrations of the somatic reflexes may, or may not, exhibit viscero-motor reflexes. The state of the visceral arcs can be estimated only by stimulating the afferent neurones of the arcs themselves.

The non-reactivity of the decapitate animal may be partly due to the artificial ventilation. Ventilating with 5% CO_2 in air was accompanied by a small increase in the amplitude of the limb movements, suggesting that hypocapnia is in part responsible for the state of the reflex arcs. The difference between the decapitated and the decapitated-chordotomized animals suggests that in the decapitated animal the cervical segments suppress the activity of lower segments of the cord. Severing the cervical region from the lower regions is accompanied by a stronger and more sensitive reflex movement. The influence of the level of transection is shown also, by the condition of the scratch reflex in various preparations. In the decapitate animal the reflex was strong and easily elicited within 3 hr. On the other hand, when the spinal cord was sectioned in the upper dorsal region there was practically no recovery of the reflex within the same time. In some way severing the cord at this level releases the viscero-motor reflexes but retards the recovery of the scratch reflex.

Relation between limb movements and blood-pressure changes. The blood-pressure changes are not passive effects dependent upon the limb movements. Blood-pressure rises may be observed in the absence of any visible movement of skeletal muscles. This is a common finding in cats in which the viscero-motor arcs are depressed, e.g. decapitates. Again, vigorous active movements of the legs did not influence the blood pressure. This was shown by those preparations in which squeezing the scrotum or labia provoked vigorous movements of the legs without change of blood pressure, whereas pinching the small intestine produced no limb movement with a rise of blood pressure. Finally, pressure rises were observed in preparations in which curarine chloride (Burroughs Wellcome) was injected intravenously in doses sufficient to paralyse respiration and abolish all visible reflex movements of the hindlimbs.

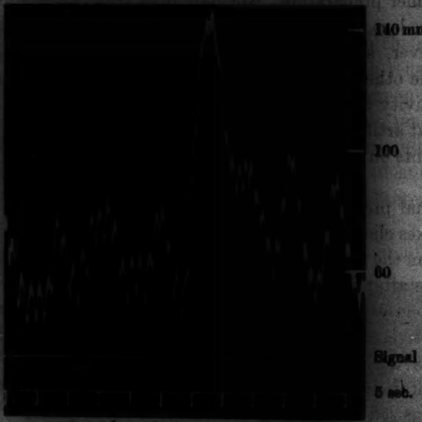

Fig. 4. Record of carotid blood pressure in decerebrate cat with spinal cord transected at C_8/T_1. Squeezing scrotal sac, excluding testis, at first signal, evoked vigorous flexion-extension movements of the hindlegs without significant change of blood pressure. Pinching ileum, at second signal, evoked a weak extension at the ankle joint with rise of blood pressure.

The pressure change was accompanied by a pallor of the intestine. This pallor appeared as the blood pressure was rising and disappeared as the pressure fell. Not only was the stimulated loop of intestine involved, but the pallor extended throughout the small intestine and into the upper one-third of the large intestine, including the caecum. This vaso-constriction is clearly an active process and is probably responsible for the major part of the change of blood pressure.

General influences. The sensitivity of the decerebrated-chordotomized preparations to visceral stimulation varied considerably from animal to animal, and even in the same animal from time to time. The majority gave good movements to gentle stimulation of the intestine, but a few did not respond, or gave only the smallest movement of the foot, to the hardest possible squeeze of the intestine between finger and thumb. No reason for this variation can be given. The impression was gained that blood loss and prolonging the time of ether inhalation would tend to give non-reactive preparations; sometimes, however, apparently similar operative procedures would give dissimilar preparations.

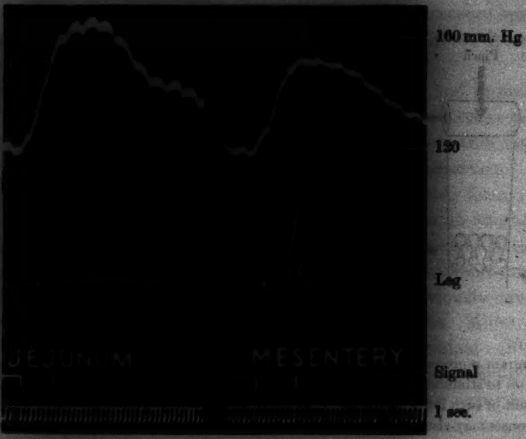

Fig. 5. Record of carotid blood pressure and movements of hindleg of a decerebrated-chordotomized cat, showing results of deterioration of preparation. Leg movement obtained on rubbing jejunal mesentery, but not on pinching jejunum. Rise of blood pressure in both instances.

The different preparations may be grouped according to their reactions to various stimuli. (1) The best preparations responded to non-traumatizing stimuli of the small or large intestine with leg movements and rise of blood pressure. (2) In the next group, the leg movements were deteriorating. Strong stimulation of the intestine produced no movements or but weak movements of the legs, but a rise of blood pressure was recorded; stimulating the mesentery or the pancreas produced vigorous leg movements and rise of blood pressure (Fig. 5). (3) In this group, intestinal stimulation severe enough to rupture the muscle layers did not elicit leg or vascular responses, but both responses were still obtained from the mesentery and pancreas. (4) The worst preparations were insensitive to stimulation of intestine, mesentery or pancreas, but did give leg movements and a rise of blood pressure on faradizing or pinching the central end of a cut splanchnic nerve.

The reactivity of an animal varied during the experimental period. When the belly was first opened some animals were relatively insensitive to intestinal stimulation, but improved if left for an hour; the majority of animals were as sensitive when the belly was opened as at any subsequent time. Repeated stimulation of the intestine, with the same strength of stimulus, at intervals of 5 to 10 min., produced leg movements and blood-pressure responses which varied in size. Occasionally a stimulus might fail entirely to produce responses, but the next stimulus might be effective. The lack of constancy in the response makes difficult any quantitative study involving successive stimulations of the gut. Prolonged experimentation, e.g. frequently eliciting viscero-motor reflexes for an hour, was often accompanied by deterioration of the responses even to extinction. Handling the intestine was another way of causing a rapid deterioration of the reflex responses, while exposing the splanchnic nerve by the retroperitoneal approach caused such profound and prolonged depression that the effects of section of the nerve could not be investigated in an acute experiment.

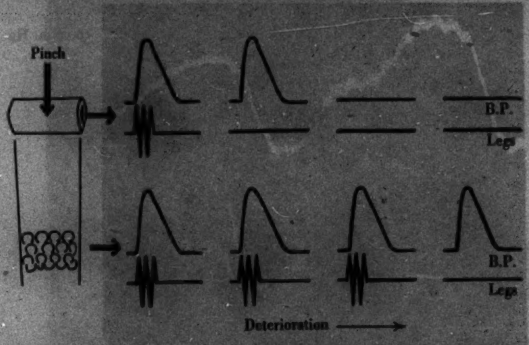

Fig. 6. Diagram representing the sequence of disappearance of the vascular and skeletal muscle responses to stimulation of the intestine and pancreas by pinching. This deterioration may be the result of such influences as bodily trauma, or handling the viscera. The different patterns of responses may also be the consequence of transecting the central nervous system at different levels.

When an animal was deteriorating, it passed progressively through the stages described above (Fig. 6). Leg responses to intestinal pinching became more difficult to elicit, and were then lost; at this stage the stimulus still elicited vascular responses. Subsequently all the responses from the intestine were absent even on crushing the muscle layers, but leg and vascular responses could be evoked from the pancreas. With further deterioration the leg and then the vascular responses were lost. By now, pinching the splanchnic nerves produced poor limb movements and but small pressure changes. All this deterioration was unaccompanied by any apparent change in the general limb reflexes; flexion, crossed extension and stretch reflexes retained their sensitivity and briskness.

The depression of the viscero-motor reflex arcs was not related to the way in which the spinal cord was cut. The decapitate animal, prepared by pithing the head, was comparable in its properties to the spinal animal prepared by the method of McDowall (1942). Cutting the cord by tying a thread tightly round the spinal dura mater, thereby conserving the normal cerebro-spinal fluid surround, did not produce unusually sensitive preparations of the decerebrated-chordotomized type.

The depression was not due to impulses from the skin and muscles which were cut when the belly wall was incised. Infiltrating these tissues with a 1% solution of procaine, so that pinching the wound edge did not cause contraction of adjacent muscle, did not alter the reactivity of the preparations.

As stated above, the pattern of the leg movements varied from animal to animal. In some the dominant movement might be flexion, in another extension, and in the majority a rhythmic stepping was superposed upon this movement. It was found also that the pattern of the movement might vary unpredictably during an experiment, an animal which previously gave flexion movements now giving extension movements, or vice versa. Miller & Waud (1925*c*) suggested that irritation at the cut surface of the spinal cord was the cause of this variation, and claimed that faradizing this area caused reversal of the movement. This claim was not substantiated in our experiments. A pledget of cotton-wool soaked in 0·9% sodium chloride solution was placed on the cut end of the thoracic spinal cord and used as one electrode for faradizing the cord face, a large pad of cotton-wool soaked in 0·9% sodium chloride solution forming an indifferent electrode. Faradic current did not cause reversal of the direction of movement of the viscero-motor reflex. The only change seen was a reduction of amplitude of the movement when the stimulation was strong enough to cause the legs to be held rigid in extension.

Sensory field of the reflexes. This field is very large. It includes not only the intestine but also the peritoneum and nearly all of the organs of the abdomen. Limb movements and blood-pressure responses have been elicited by stimulating all regions of the small and large intestine by pinching. Pinching the pancreas, ovaries, uterine horns, spleen and body of the stomach at the greater curvature was effective, and also pulling on the intestinal mesentery, ovaries and splenic ligaments. Pinching or faradizing the nerves running in the intestinal mesentery, and the central end of a cut splanchnic nerve as it crosses the crus of the diaphragm, also elicited responses.

Rubbing the mesentery lightly with a dry wool pellet, and rubbing the surface of the parietal peritoneum lining the abdominal wall, produced leg movements and blood-pressure changes. It was noted that in some animals, however, the parietal peritoneal stimulation was ineffective, whereas the mesentery was still sensitive.

Repetition of visceral stimuli. If the reflex movements of the limbs were elicited by repeated stimulation, the response diminished in size and was ultimately lost. This decay was seen if the interval between the stimuli was short, of the order of 5–20 sec., and generally only three to five responses were obtained. If the preparation was left for 5 min. the responses reappeared and showed the same decay as before, if again elicited repeatedly. This feature of the reflex was seen both with pinching the intestine or with faradizing the mesenteric nerves.

The results of repeated stimulation included a loss of response from immediately adjacent lengths of intestine, but not from more distant loops of gut. Thus, with repeated pinching of the intestine in two places about half an inch apart leg movements disappeared. Pinching the intermediate piece of intestine, although it was previously unstimulated, was now without effect. On the other hand, pinching a neighbouring loop of the intestine was still effective. Similar local loss of sensitivity was seen when the mesenteric nerves were stimulated directly. Repeated electrical stimulation of a nerve resulted in diminishing leg movements, and ultimate loss of response; stimulation of a neighbouring nerve, however, still elicited responses. It may be remarked at this juncture that the intestine often showed a patchy sensitivity to stimulation, severe crushing of one loop being ineffective, whereas gentle pinching of a neighbouring loop was effective. Sometimes the insensitive regions were quite short, of the order of 2–3 cm.

It is not possible to state where the change in the reflex arcs took place in these experiments, although it can be inferred that the spinal motor neurone itself was not affected. The observations could be explained by either a change in the spinal connector-neurone path or by an altered state of the afferent pathways themselves between the intestine and spinal cord. It is conceivable that repeated stimulation of visceral afferent fibres results directly or reflexly in a reduction of their excitability or conductivity. To settle the question, some more direct approach to the problem is needed. At present the situation may be best summed up in the words of Sherrington (1906): 'The interoceptive arcs appear in most segments less closely connected with the skeletal musculature than with the visceral.'

Anatomy of the reflex arcs. The evidence of other workers shows that the splanchnic nerves form a great afferent pathway from the intestine. The vagus nerves are not parts of the reflex arcs involved. Sectioning the vagus-sympathetic trunks in the neck leaves the reflexes intact; the only effect is a heightening of the blood-pressure responses, presumably because of loss of the last remaining pathway for effective compensatory adjustments.

Mediation of the limb and vascular reflexes by thoracic-lumbar-sacral segments of the cord, with all connexions with the upper brain stem severed, shows that the reflex arcs are completed within the cord. The efferent paths carry vaso-constrictor impulses to the vessels of the intestine, presumably via the splanchnic nerves, and motor impulses to the muscles moving the lower limbs and belly wall via lumbar and sacral nerves. It is appreciated that the adrenal glands may have some responsibility for the vascular responses, and the mechanism and anatomy of the response requires fuller investigation.

DISCUSSION

It is clear that impulses from the abdominal viscera can influence skeletal as well as vascular musculature. In appropriate preparations mechanical stimulation of the intestine elicits the viscero-motor and the viscero-visceral reflexes together, and the presence of the reflexes in the animal with the cord cut in the upper thoracic region shows that the arcs are completed within the spinal cord below that level. The reflexes are obtained not only from the intestine, but also from the intestinal mesentery, the pancreas and other abdominal organs. Other investigators have shown that the splanchnic nerves form the way-in for impulses from the majority of the abdominal viscera, and the elicitation of the reflexes by stimulating the bared mesenteric and splanchnic nerves suggests that these are the afferent pathways of the specific arcs which have been studied. The vascular reflexes are not dependent upon the vagus nerves; if anything, dividing the nerves heightens the blood-pressure changes, presumably by destruction of reflex buffer mechanisms.

The skeletal muscle responses consist of movements of the hindlegs and contraction of the abdominal muscles. The leg movements vary considerably in their pattern, and, for example, may even change from flexion to extension in the same animal during the experiment. The finding of Miller & Waud (1925*c*), that this is due to irritation at the cut face of the spinal cord, was not confirmed in the experiments, but no other explanation can be offered. The vascular response consisted of a rise of general arterial pressure, which was accompanied by vaso-constriction of a wide area of the intestine. Sherrington (1906) pointed out that conduction is more easily depressed through long arcs in the spinal cord than through short arcs. This is borne out by these experiments. The skeletal muscle responses disappeared before the vascular responses, so that a preparation might show only a blood-pressure response to intestinal stimulation, with no leg or abdominal movements. This dissociation of the arcs depended not only upon certain general influences but also upon the level of transection of the brain stem. The decerebrate animal, with the brain stem transected at the intercollicular level, is characterized by its lack of reaction to stimulation of any of the viscera; rise of blood pressure was the exception rather than the rule. When the level of transection is below the medulla, reflex responses are obtained on visceral stimulation. Transection in the upper cervical regions gives a preparation in which the viscero-motor reflexes are usually weak in execution, and elicited only by strong stimulation of the intestine; the responses may be absent when the abdomen is first opened, as found by Lewis & Kellgren (1939). Transection in the upper thoracic segments gives a preparation in which the viscero-motor reflexes are stronger and can be elicited by gentle stimulation of the intestine. In both spinal preparations pinching the intestine caused a rise of blood pressure, and pinching the pancreas or stimulating the mesentery produced both leg and blood-pressure responses. One explanation of the variations in the conductivity of the spinal arcs in the preparations would be an active suppression of the reflexes by higher levels of the neuraxis. It must be remembered, however, that the nervous system is very sensitive to changes in its blood supply, and there is no information about the nutrition of the spinal arcs in the various preparations. On the other hand, it would be expected that altered nutrition would affect all reflex arcs in the cord, and it is found that the state of the reflexes elicited by visceral stimuli is not related to the state of the reflexes elicited by skin stimulation, although the same final common path is involved in each case.

At present the decerebrated-chordotomized animal, with the cord cut in the upper thoracic region, seems to be the most suitable preparation for investigating the viscero-motor and allied reflexes in the acute experiment. Not only does the preparation maintain its own respiration, but the intestine is more sensitive and the reflexes are stronger. It must be admitted, however, that in any preparation the reflexes are very sensitive to any deleterious influence such as excessive trauma or haemorrhage. Also the level of sensitivity of the reflexes varies unpredictably, which makes it impossible to obtain a constant response for any length of time and limits the scope of experiments.

In any preparation, deterioration is shown by a loss of responses to intestinal stimulation, although stimulating the pancreas or mesentery is still effective. With further deterioration, stimulating the pancreas or mesentery ceases to be effective, and to obtain responses one must stimulate the splanchnic nerve directly. Thus, a preparation which gives leg responses on pinching the intestine and pancreas may change into one giving no leg movements on pinching the intestine, but giving leg movements on pinching the pancreas; on every occasion blood-pressure rises may be recorded. It is clear, therefore, that the absence of leg movements on pinching the intestine does not signify the anatomical absence of a certain type of innervation of the intestine. This point is stressed because Lewis, as a result of his experimental findings, considered that the intestine lacks a functional type of nerve fibre which is present in the mesentery and pancreas. The similarity of response on pinching the intestine, mesentery and pancreas in suitable preparations suggests that the intestine, mesentery and pancreas are supplied by afferent fibres whose impulses can have the same functional significance within the central nervous system. What does differ between the innervations of the viscera is the stability of the spinal reflex in face of certain general influences or the action of higher levels of the nervous system.

Lewis (1942) has recently suggested that the sensory fields of the body could conveniently be grouped into a superficial and a deep field, the latter including skeletal muscles and also the mesentery with its contained pancreas. Each of the fields is supplied by systems of nerve fibres which carry impulses producing a sensation of pain on arrival in the central nervous system. The intestine was excluded from the deep field because of the apparent absence of the afferent limb of the viscero-motor arc, Lewis considering that this was further evidence of lack of a pain-producing afferent innervation of this organ. There is evidence, however, that animal experiments do not substantiate the latter claim, the presence or absence of viscero-motor reflexes on pinching the intestine depending upon the method of preparation of the animal. If the older division of the sensory fields by Sherrington were to be discarded, it is clear that the intestine is not entirely lacking in afferent innervation and would need to be included in the deep field of Lewis.

It is appreciated that in the acute experiment the spinal reflex arcs are in a condition of spinal shock. It is difficult to assess the effect of this shock upon the reflexes which have been studied. The changes in the strength and sensitivity of the reflexes may be an expression of alterations in the degree of the shock, and it is also possible that some other factor may be acting on the synapse or conducting fibre. In any case, it is probable that the condition of the spinal neurones would lower the conductivity of all the spinal arcs, and the ready dissociation of the responses may, in great measure, be due to this.

SUMMARY

1. Reflex movements of the hindlegs and rises of blood pressure have been recorded after visceral stimulation, particularly after pinching the intestine. The reflex arcs are complete within the spinal cord.
2. The reflexes are strongest and most easily elicited in the decerebrate animal with the spinal cord cut in the upper thoracic level.
3. The reflexes are weaker, and movements may be absent, upon intestinal stimulation in the decapitate animal.
4. Movements are absent, but blood-pressure changes have been recorded, on pinching the intestine of the decerebrate animal.
5. The viscero-motor reflexes may be lost while the viscero-vascular reflex remains. This indicates changes in the spinal cord.

6. When intestinal stimuli are ineffective, powerful reflex responses follow stimulation of the mesentery and pancreas.

7. Changes in conductivity in the visceral reflex arcs occur without changes in the somatic reflex arcs.

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HYPERPNOEA INDUCED BY FORCED BREATHING

By [illegible]

From the Department of [illegible]

(Received [illegible])

The absence of apnoea after forced breathing has hitherto been [illegible] is not [illegible] of the chemical control of respiration. [illegible] subjects, found hyperpnoea instead of apnoea, but [illegible] any cause for the phenomenon. He suggested that the hyperpnoea might be due to anoxia following [illegible] and recorded that the systolic blood pressure fell 20 mm. Hg as a result of forced breathing, whereas the blood pressure of Dr [illegible] who showed apnoea after forced breathing, hardly fell at all. [illegible] that this problem might be worth further investigation.

METHODS AND SUBJECTS

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[illegible] reflex responses follows [illegible] and [illegible].

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HYPERPNOEA INDUCED BY FORCED BREATHING

By J. N. Mills

From the Department of Physiology, University of Oxford

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The absence of apnoea after forced breathing has frequently been recorded, but seems to have received little attention, presumably since it appears contrary to accepted ideas of the chemical control of respiration. Boothby (1912), with himself as subject, found hyperpnoea instead of apnoea, but did not succeed in demonstrating any cause for the phenomenon. He suggested that the hyperpnoea might be due to anoxia following diminished medullary circulation, and recorded that his systolic blood pressure fell 20 mm. Hg as a result of forced breathing, whereas the blood pressure of Dr Haldane, who showed apnoea after forced breathing, hardly fell at all. With the increasing recognition of a variety of nervous factors in respiratory control it appeared that this problem might be worth further investigation.

METHODS AND SUBJECTS

The subjects were mainly medical students and laboratory workers, all apparently in good health but of very varying degrees of athletic ability and training. They were usually examined recumbent on a surgical couch with the shoulders raised, after a preliminary rest of at least 15 min. They were kept as far as possible in ignorance of the object and expected result of the experiments, which were never discussed with them until complete.

Respiratory recordings were obtained with a chest stethograph connected to a tambour and ink writer. When quantitative measurements of ventilation were required, the subject breathed through a mouthpiece and rubber or mica disk valves, and the expired air was collected in a bag of suitable size, a small 5 l. bag being used for short experiments. The volume was measured with a Bohr wet meter. When the subject was inspiring air of abnormal composition this was supplied from a bag. The magnitude of the ventilation during forced breathing was determined by measuring the volume of air left in the bag, after a measured period of forced breathing, and subtracting this volume from the volume originally present in the bag. The same procedure was sometimes adopted when the subject was inspiring room air. All results are expressed in l./min. corrected to s.t.p. dry.

Alveolar samples were end-inspiratory Haldane-Priestley samples except for the resting controls, which were the mean of three pooled end-inspiratory and three pooled end-expiratory samples. Gas analyses were performed in duplicate with the Haldane apparatus.

Blood pressure was measured with a Riva-Rocci cuff by the auscultatory method. To detect the fluctuations during the different phases of respiration, the cuff pressure, after being raised above the maximum systolic pressure, was gradually lowered until, at a certain pressure, audible beats came through at only one phase of respiration, at first singly. The phase and pressure were noted.

The pressure was then gradually lowered further. Longer series of beats now came through with shorter intervals between the groups. The phase of respiration at which one or two beats were missed was noted, as also the pressure at which the beats first came through regularly. In this manner maximal and minimal systolic pressures were obtained, corresponding to known phases of respiration. It was sometimes possible to obtain two values for the diastolic pressure by a similar procedure.

Frontal, parietal and occipital leads were connected to a three-channel Grass amplifier of usual type to obtain electroencephalograph records.

The statistical techniques employed have been those of Fisher (1941). The conventional sense of 'significant' ($P<0{\cdot}05$) and 'highly significant' ($P<0{\cdot}01$) has been used.

RESULTS

Subjects were instructed to overbreathe as hard as they could, at whatever frequency they found most convenient, usually between 20 and 30 respirations per min. This standard procedure, within 1–1½ min., reduced the alveolar CO_2 tension to about 20 mm. Hg and raised the alveolar O_2 tension to about 135 mm. Hg. When the subject was connected to mouthpiece and valves for the forced breathing, he was instructed to breathe only as hard as was possible without becoming conscious of any resistance from the valves. It was, of course, only in these experiments that the magnitude of the voluntary overventilation could be measured, and in seventy-one such determinations upon eight subjects values of between 50 and 70 l./min. were usually found. When mouthpiece and valves were not used the overventilation was probably more vigorous.

Of thirty-five subjects selected at random only ten showed an apnoea fairly consistently after 1 or 1½ min. of forced breathing. Eight subjects, on the contrary, appeared breathless after forced breathing, and continued to overventilate involuntarily, often with great vigour. The remaining seventeen showed an intermediate picture; some continued breathing, though not very vigorously, whilst some were completely inconsistent in their behaviour despite every effort to control modifying factors. It is possible that some of this variability is due to the unfamiliar experience of not wanting to breathe; and in fact three subjects, who at first showed some continuance of breathing after forced breathing, became consistently apnoeic with frequent repetition of the experiment. Other subjects, however, became less consistent in their behaviour with frequent repetition.

This study is mainly concerned with four subjects who were regularly hyperpnoeic after forced breathing and with two others whose behaviour was similar and who were discovered during class-work, namely, J.M.H.B., J.E.C., E.T.G., L.D.H., J.D.A. and J.C.S.A.W. These are referred to for convenience as 'hyperpnoeic subjects'. Upon these six subjects a total of about 200 forced breathing experiments have been performed. The four others, who behaved in a similar manner, were not available for frequent experiments, and the six were not always available as often as seemed desirable.

The magnitude and form of the hyperpnoea after forced breathing varied from subject to subject. The most usual form was that shown by Boothby, rapid respiration of gradually diminishing depth. Some subjects, however, went on overventilating at undiminished depth for some minutes, whilst others at times merely gave two or three deep breaths or a series of slow deep breaths. Typical examples are shown in Fig. 1, and average magnitudes in Table 1.

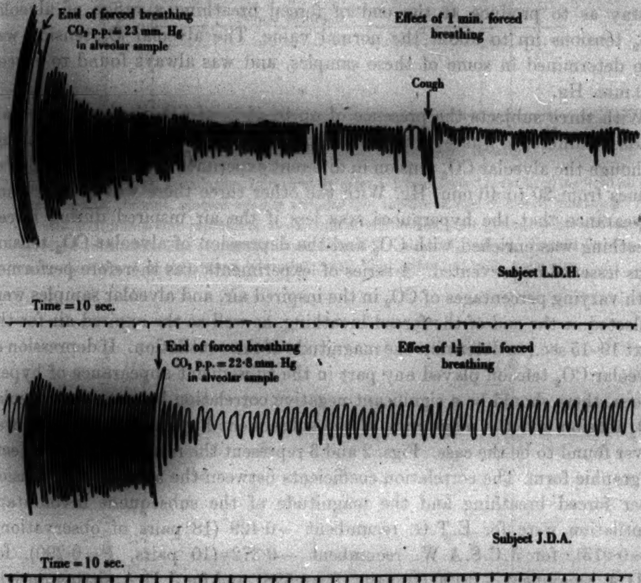

Fig. 1. Stethograph records showing involuntary hyperpnoea after forced breathing. Arrow indicates end of forced breathing. Inspiration downwards.

The possible causes for this hyperpnoea that have been investigated are:

(1) Acapnia produced by overventilation, as was apparently tacitly assumed by Boothby.

(2) The giving of an alveolar sample at the end of forced breathing.

(3) A fall of blood pressure, which Vincent & Thompson (1928) and others have found to accompany forced breathing.

(4) Increased venous return consequent upon the forcible respiratory movements.

(5) Impairment of cortical function, or some cortical activity independent of impaired function.

Acapnia. The six subjects who were regularly hyperpnoeic after a period of forced breathing were made to do similar spells of forced breathing inspiring from a bag containing either room air or air to which varying amounts of CO_2 had been added. Alveolar samples were collected at the end of forced breathing to determine how far the usual depression of CO_2 tension had been prevented. From the experience of the first series of such experiments on any subject, the composition of the air in the bags was chosen for further experiments in such a way as to produce at the end of forced breathing a range of alveolar CO_2 tensions up to about the normal value. The alveolar O_2 tension was also determined in some of these samples, and was always found to exceed 100 mm. Hg.

With three subjects the presence of up to 4½% of CO_2 in the inspired air made no difference whatever to their respiratory response to forced breathing, although the alveolar CO_2 tension in different experiments occupied a range of values from 20 to 40 mm. Hg. With the other three there was at first some appearance that the hyperpnoea was less if the air inspired during forced breathing was enriched with CO_2 and the depression of alveolar CO_2 tension thus lessened or prevented. A series of experiments was therefore performed with varying percentages of CO_2 in the inspired air, and alveolar samples were collected at the end of the forced breathing, as well as the expired air for the next 10–15 sec., to determine the magnitude of the ventilation. If depression of alveolar CO_2 tension played any part in the subsequent appearance of hyperpnoea, there should be a significant negative correlation between the alveolar CO_2 tension and the magnitude of the subsequent hyperpnoea, but this was never found to be the case. Figs. 2 and 3 represent the results on two subjects in graphic form. The correlation coefficients between the alveolar CO_2 tension after forced breathing and the magnitude of the subsequent involuntary ventilation were for E.T.G. recumbent $-0{\cdot}429$ (18 pairs of observations, $P=0{\cdot}075$), for J.C.S.A.W. recumbent $-0{\cdot}372$ (10 pairs, $P=0{\cdot}290$), for J.D.A. recumbent $+0{\cdot}190$ (10 pairs, $P=0{\cdot}598$), and for J.D.A. sitting upright $-0{\cdot}197$ (10 pairs, $P=0{\cdot}586$). The P values in each case indicate the probability of obtaining such a result if the two variables were uncorrelated. It will be seen that no correlation reaches the conventional level of significance, so there are no grounds for asserting any connexion between the acapnia induced by forced breathing and the subsequent involuntary hyperpnoea. Figs. 2 and 3 show, in addition, that considerable hyperpnoea can follow forced breathing even if acapnia is minimal or absent.

A further confirmation that the hyperpnoea which follows forced breathing is not due to moderate acapnia was obtained on E.T.G. He was made to increase his breathing slightly for 10–20 sec., reducing his alveolar CO_2 tension to 30–35 mm. Hg. The subsequent ventilation was almost normal. By contrast, after vigorous forced breathing from a bag containing 2–5% CO_2, ending up

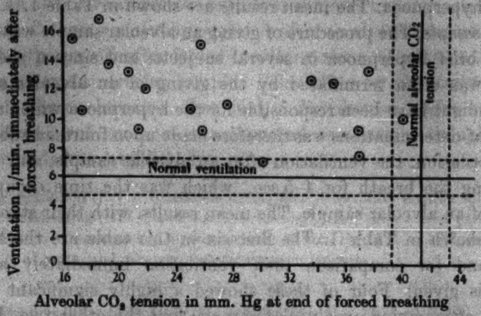

Fig. 2. Subject E. T. G. Ventilation, immediately after 1–2 min. forced breathing, inspired air with varying CO_2 content, plotted against alveolar CO_2 tension at end of forced breathing. Horizontal continuous line represents mean control ventilation after giving an alveolar sample; vertical continuous line represents mean control alveolar CO_2 tension. Dotted lines in each case show E.S.D. of individual determinations. Ventilation in l./min. S.T.P. dry.

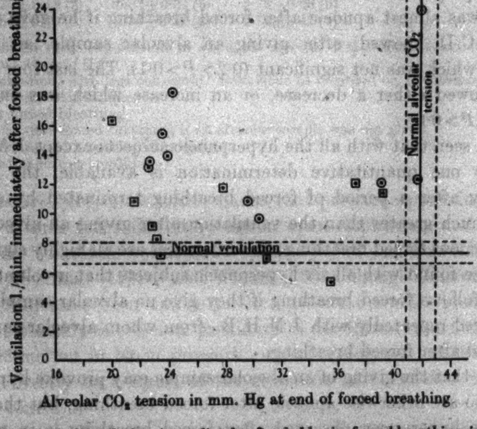

Fig. 3. Subject J. D. A. Ventilation, immediately after 1–1½ min. forced breathing, inspired air with varying CO_2 content, plotted against alveolar CO_2 tension at end of forced breathing. Horizontal continuous line represents mean control ventilation after giving an alveolar sample; vertical continuous line represents mean control alveolar CO_2 tension, both taken when subject was recumbent. Dotted lines in either case show E.S.D. of individual determinations.

⊙ Subject recumbent on couch. ⊡ Subject sitting upright. Ventilation in l./min. S.T.P. dry.

with an alveolar CO_2 tension of 30–40 mm. Hg, there was a considerable involuntary hyperpnoea. The mean results are shown in Table 1.

Alveolar sample. The procedure of giving an alveolar sample was observed to provoke a brief hyperpnoea in several subjects, and since a spell of forced breathing was often terminated by the giving of an alveolar sample, this procedure might have been responsible for the hyperpnoea which occurred.

A series of determinations was therefore made upon fourteen subjects of the resting ventilation, the ventilation after an alveolar sample, and usually also after holding the breath for 4–5 sec., which was the time occupied by the collection of an alveolar sample. The mean results, with their standard deviations, are shown in Table 1. The first six in this table are the hyperpnoeic subjects, and for comparison their ventilation immediately after forced breathing is given. Four of these showed a highly significant increase in ventilation after giving an alveolar sample, and the other two, E. T. G. and J. D. A., an increase which was not quite significant ($0{\cdot}2 > P > 0{\cdot}05$). Of the eight subjects who were not regularly hyperpnoeic after forced breathing, F. A. de H., C. H. C and W. W. S. showed a highly significant increase in respiration after giving an alveolar sample, whether comparison be made with the resting ventilation or with the ventilation after 4–5 sec. of breath-holding. W. W. S., after a period of forced breathing terminated by an alveolar sample, showed a hyperpnoea almost identical with that after giving an alveolar sample alone, but was almost apnoeic after forced breathing if he gave no alveolar sample. C. C. D. showed, after giving an alveolar sample, an increase in respiration which was not significant ($0{\cdot}2 > P > 0{\cdot}1$). The last four subjects in Table 1 showed either a decrease, or an increase which was entirely non-significant ($P > 0{\cdot}6$).

It will be seen that with all the hyperpnoeic subjects except J. M. H. B., for whom only one quantitative determination is available, the ventilation immediately after a period of forced breathing terminated by an alveolar sample is much greater than the ventilation after giving an alveolar sample without previous forced breathing; the differences are all highly significant. It has also been found with all six hyperpnoeic subjects that involuntary hyperpnoea still follows forced breathing if they give no alveolar sample. This has been observed repeatedly with J. M. H. B., from whom alveolar samples were not collected after forced breathing.

It is clear that the giving of an alveolar sample may provoke hyperpnoea in subjects who are otherwise apnoeic after forced breathing, but that in many subjects the hyperpnoea observed after forced breathing is in no way dependent upon the giving of an alveolar sample.

It does appear, however, that those subjects who are habitually hyperpnoeic after forced breathing are more likely to show hyperpnoea after giving an alveolar sample than are those who are apnoeic after forced breathing. If we ignore the three uncertain subjects, who showed a nearly significant hyperpnoea after an alveolar sample, we have four subjects hyperpnoeic after forced breathing, all of whom are also hyperpnoeic after an alveolar sample, and seven subjects not hyperpnoeic after forced breathing alone, of whom only three are hyperpnoeic after an alveolar sample. Calculation by the exact method of Fisher (1941) shows, however, that such a distribution might be found by chance in 10·61 % of trials, so the supposed association, though probable, cannot be regarded as established.

TABLE 1. Resting ventilation (R.V.); ventilation during first 15 sec. after alveolar sample (A.S.); after 4–5 sec. of breath-holding (B.H.); after forced breathing (F.B.); after a brief period of respiratory increase sufficient to reduce alveolar CO_2 tension to 30–35 mm. Hg (M.H.). All figures in l./min. S.T.P. dry, with E.S.D. of mean, and number of observations in brackets

Subject	R.V.	A.S.	B.H.	F.B.	M.H.
L. D. H.	7·44 ± 0·51 (6)	13·36 ± 0·40 (4)	8·75 ± 0·61 (4)	28·74 ± 1·30 (7)	
E. T. G.	5·37 ± 0·10 (6)	6·11 ± 0·26 (23)		11·77 ± 0·64 (18)*	6·04 ± 0·50 (5)
J. D. A.	6·73 ± 0·17 (6)	7·32 ± 0·19 (15)		14·35 ± 1·31 (10)	
J. C. S. A. W.	6·06 ± 0·31 (9)	7·65 ± 0·41 (9)		13·08 ± 0·82 (10)	
J. E. C.	6·34 ± 0·21 (9)	18·41 ± 1·72 (9)	7·85 ± 0·88 (4)	26·87 ± 3·68 (10)	
J. M. H. B.	7·18 ± 0·66 (12)	22·99 ± 1·92 (10)	10·70 ± 0·71 (10)	19·04†	
F. A. de H.‡	5·24 ± 0·18 (6)	8·29 ± 0·55 (5)	5·90 ± 0·18 (6)		
C. H. C.‡	4·67 ± 0·12 (8)	15·96 ± 1·53 (6)	5·27 ± 0·25 (6)		
W. W. S.§	6·39 ± 0·31 (7)	8·73 ± 0·47 (12)	6·40 ± 0·34 (7)	8·78 ± 1·00 (6)	
C. C. D.‖	6·90 ± 0·32 (8)	8·42 ± 0·72 (6)	7·03 ± 0·37 (6)		
J. N. M.‡	6·48 ± 0·16 (17)	6·98 ± 1·56 (9)			
M. W. G.‖	6·16 ± 0·16 (12)	6·25 ± 0·19 (17)			
T. J. M.‡	5·25 ± 0·18 (8)	5·02 ± 0·27 (6)	5·79 ± 0·28 (6)		
R. P. C. H. J. ¶	6·84 ± 0·20 (6)	7·36 ± 0·22 (4)	8·14 ± 0·42 (4)		

* 10·97 ± 1·00 (6) for those experiments in which the alveolar CO_2 tension at end of forced breathing was between 30 and 40 mm. Hg.

† Single determination, with legs congested. Behaviour as recorded on the stethograph was always much the same. No alveolar sample.

‡ Apnoeic after forced breathing.

§ Almost apnoeic after forced breathing if an alveolar sample was not given.

‖ Behaviour after forced breathing inconsistent.

¶ Hypopnoeic after forced breathing.

Blood pressure. Heymans (1929) claims that pressoreceptors in the carotid sinus exert a tonic reflex inhibitory action upon respiration, and that fall of blood pressure will therefore act as a respiratory stimulus. This claim has been borne out by Schmidt (1932*a*) and Winder (1938), though Galston, Goldstein & Steele (1943) have been unable to demonstrate a similar phenomenon in man, probably owing to the close proximity of carotid sinus and carotid body. A sudden reduction in blood pressure may also (Schmidt, 1932*b*) stimulate respiration directly as a result of the reduction of blood flow through the respiratory centre.

It seemed worth determining whether, in the subjects with different respiratory reactions after forced breathing, any characteristic difference existed in the behaviour of their blood pressure, as was supposed by Boothby (1912). Measurements were therefore made of the systolic pressure in the brachial artery. The size of the respiratory fluctuation and the phase of respiration when the maximum and minimum pressure occurred were noted, and these measurements were repeated during and after forced breathing. A few readings of diastolic pressure were also obtained, but, owing to the difficulty of obtaining a series of readings when the respiratory fluctuations were large and the pressure changing rapidly, not much weight can be attached to these, and they will not be further considered. For these experiments I have chosen subjects showing each type of respiratory behaviour after forced breathing, three who were hyperpnoeic, four who were apnoeic, and three who were intermediate. In all, thirty-five sets of measurements were made, excluding some early ones before the technique was satisfactory.

The most consistent finding during forced breathing was a fall of as much as 60 mm. Hg in the systolic pressure at the height of inspiration, with a smaller drop, or even a slight rise, in the expiratory systolic pressure. The diastolic pressure usually altered in the same direction as the systolic, but the changes were smaller. During forced breathing, therefore, the systolic pressure was lower during inspiration than during expiration in all but three experiments. The difference was most commonly between 10 and 20 mm. Hg, but, in four experiments, was greater than 30 mm. Hg. The change, after the forced breathing was over, varied both from subject to subject and in the same subject. The systolic pressure sometimes returned to normal at once; at other times it rose to 40 mm. Hg above the resting level, or remained down to 18 mm. Hg below it, but usually returned to normal in about 3 min.

There was no consistent relationship between the behaviour of the blood pressure and of the respiration. As large a fall of systolic pressure was observed during the forced breathing in subjects subsequently apnoeic as in those subsequently hyperpnoeic; and a subsequent rise or fall was observed in subjects in each group. Some subjects showed repeatedly the same respiratory behaviour, although their blood pressures behaved differently on different occasions. A pressure remaining slightly below the initial resting level when the forced breathing was complete was somewhat more frequent in those subjects who were hyperpnoeic than in those who were apnoeic; but it is at least as probable that this is a consequence as that it is a cause of the continued hyperpnoea.

The most obvious ways in which reduction of blood pressure could affect respiration are by reflex action from the carotid sinus, and by direct chemical effect upon the respiratory centre through a reduction in the blood flow. In either case the changes would be exaggerated if the head were above the level of the heart and minimized if it were below it. A series of twenty-three measurements of ventilation after forced breathing was therefore made upon three of the hyperpnoeic subjects seated upright in a chair with the head supported by cushions, and, for comparison, a series of measurements of resting ventilation in this position was also made. The figures obtained are shown in Table 2, with the corresponding figures obtained in the usual recumbent position. It will be seen that, with each subject, the ventilation after forced breathing was less when the subject was sitting upright than when he was recumbent, although only with J.D.A. was the difference significant ($0{\cdot}02 > P > 0{\cdot}01$). The resting ventilation of this subject was somewhat less when he sat upright than when he was recumbent, and the difference between ventilation after forced breathing and the resting ventilation was not in fact significantly different in the two positions. There is therefore no support for the suggestion that a reduction of cerebral blood pressure or flow might contribute to the hyperpnoea.

The converse experiment has been performed upon two regularly hyperpnoeic subjects. They lay on a tilting stretcher with the head end inclined downwards at an angle of 13°, and sphygmomanometer cuffs were inflated round both thighs at a pressure of about 90 mm. Hg to minimize any increase in venous return due to this position, since such an increase sometimes stimulates respiration (Mills, 1944). Immediately (7–17 sec.) before the end of the forced breathing the head end was further lowered to an angle of 26°. This procedure should certainly have prevented any fall in carotid blood pressure, but both subjects showed hyperpnoea indistinguishable from that which they showed in the usual position. Three sets of measurements of the blood pressure during this procedure were made upon E.T.G., with the cuff round the upper arm held at or slightly above the level of the carotid sinus. During forced breathing the inspiratory systolic pressure was never more than 6 mm. Hg below its resting level, whereas in the usual position it was always reduced by at least 10 mm. Hg. The expiratory systolic pressure was always above its previous resting level, whereas in the usual position it was more often below. After completion of the forced breathing the systolic pressure was always above its initial level. When similar experiments were carried out in the usual position, the systolic pressure was lowered for a period up to 2 min. The procedure, in fact, reversed the usual fall of carotid blood pressure after forced breathing, for, in the experiments in the usual position with the head somewhat raised, the carotid pressure

TABLE 2. Mean ventilation: resting (R.V.), and during first 15 sec. after forced breathing (F.B.); with the subject sitting upright (sitting), or recumbent on couch with back propped up (lying). All figures in l./min. S.T.P. dry with E.S.D. of mean and number of observations in brackets

Subject	Position	R.V.	F.B.
J.C.S.A.W.	Sitting	6·73±0·24 (12)	11·44±0·79 (9)
	Lying	6·06±0·31 (9)	13·08±0·82 (10)
J.D.A.	Sitting	5·49±0·23 (9)	9·95±1·01 (10)
	Lying	6·73±0·17 (6)	14·35±1·31 (10)
E.T.G.	Sitting	7·09±0·21 (6)	9·94±0·59 (4)*
	Lying	5·37±0·10 (6)	10·97±1·00 (6)*

* Inspiring air with increased CO_2 content sufficient to leave alveolar CO_2 tension between 30 and 40 mm. Hg at end of forced breathing.

must, for gravitational reasons, have been lower than the recorded brachial pressure; the respiratory behaviour was, however, unaffected.

Mechanical effects of forced breathing upon cardio-vascular system. It is known that increased venous return, or congestion of the pulmonary vessels, may provoke a reflex hyperpnoea (see Mills (1944) for references), and either of these changes may result mechanically from the act of forced breathing. If an increased venous return is responsible for hyperpnoea, then the hyperpnoea should at least be decreased if the venous return is limited by venous congestion of the legs. In five of the six hyperpnoeic subjects, spells of forced breathing were performed after sphygmomanometer cuffs round both thighs had been inflated at a pressure of 80–90 mm. Hg for 5–45 min., and, in one experiment, after cuffs had also been inflated round the arms at 70–80 mm. Hg. Ebert & Stead (1940) have shown that such congestion applied to both thighs and one arm for as little as 7–10 min. will abstract about 720 c.c. of blood from that previously contained in the vessels of the head, trunk and remaining one arm, or about 15% of the volume previously present in those regions. Such abstraction of blood must limit the possible magnitude of the venous return from the remainder of the body, and a similar procedure has been used by Barcroft & Edholm (1945) as an adjunct in the production of haemorrhagic fainting. It will be seen below that this procedure can prevent a hyperpnoea which is apparently due to increased venous return. In no experiment upon these five hyperpnoeic subjects with limbs congested, however, was the involuntary hyperpnoea after forced breathing less than that usually observed upon the subject, as the figures in Table 3 show. An increased venous return induced by the forced breathing does not therefore appear to be a necessary cause of the hyperpnoeic sequel in these subjects.

TABLE 3. Ventilation during first 15 sec. after 60–80 sec. of forced breathing when venous congestion had been applied to both thighs for varying periods

Subject	Time of congestion (min.)	Ventilation (l./min.)	Composition of inspired air
J. D. A.	?	18·91	3% CO_2
J. M. H. B.	31	19·04	Room air
J. E. C.*	5	26·22	4½% CO_2
E. T. G.	30	16·39	4½% CO_2
E. T. G.	45	16·83	Room air
L. D. H.	30	31·53	Room air
L. D. H.	45	29·74	Room air

* Congestion also applied to both arms.

The mechanical effects of respiration upon the circulation are, however, more extensive than a mere increase in venous return to the right heart. Numerous workers, such as Cahoon, Johnson & Michael (1940), Heinbecker (1927), Katz & Gauchat (1924) and Shuler, Ensor, Gunning, Moss & Johnson (1942), have pointed out that the effect of inspiration in increasing diastolic filling, and hence stroke volume, of the right heart is counteracted in the left heart by the increased capacity of the pulmonary vessels during inspiration. Lewis (1908) and Battro, Segura, Elicabe & Araya (1944) have shown that, whereas thoracic inspiration causes in man a fall of blood pressure, diaphragmatic inspiration causes a rise; the suggested explanation is that diaphragmatic contraction raises the intra-abdominal pressure and so produces a big increase in diastolic filling of the right heart, whilst thoracic inspiration exerts its main effect upon the circulation by increasing the capacity of the pulmonary vessels and so lessening the return of blood to the left heart. A comparison was therefore made between the effects of forced breathing confined, so far as possible, to the thorax or diaphragm. Success in this manoeuvre was usually obvious to visual observation. When breathing was confined to the thorax, the abdominal wall was retracted during inspiration, whilst, when the diaphragm alone was used, the abdomen expanded during inspiration and the thorax remained almost stationary. The degree of thoracic expansion could be recorded with the stethograph, and the presence and direction of abdominal movement was confirmed by placing a hand lightly upon the abdomen. One subject was incapable of using his diaphragm only, but the others all succeeded after some practice.

In some experiments, forced breathing was continued for 1 min., but, in others, shorter periods, down to about 15 sec., were used, so as to produce the minimum of acapnia.

Four of the hyperpnoeic subjects examined in this way showed no consistent deviation from their customary respiratory behaviour. Upon scrutiny of the results on ten other subjects selected at random, it was found that they could be classified roughly according to the degree of athletic training of the subjects. Four subjects were in good athletic training, indulging regularly in such vigorous exertion as rowing or Rugby football, the other six were not in training and readily became breathless upon mild exertion.

The four subjects in training responded, on the whole, in a similar manner to thoracic or diaphragmatic forced breathing. One subject was, however, a little less apnoeic after diaphragmatic than after thoracic forced breathing.

The six subjects not in athletic training all showed characteristic differences in their respiratory behaviour after forced breathing performed with thorax or diaphragm. Three were apnoeic after thoracic forced breathing but, after diaphragmatic forced breathing, showed hyperpnoea, as illustrated in the stethograph tracings in Fig. 4. One subject continued breathing gently after thoracic forced breathing but was obviously hyperpnoeic after diaphragmatic forced breathing. One, hypopnoeic after either procedure, asserted that, after the thoracic forced breathing, he felt no compulsion to breathe as he did after diaphragmatic, even though the forced breathing was performed for a longer period with the diaphragm than with the thorax. The sixth subject was apnoeic after either procedure, but, whereas 1 min. periods of thoracic or unrestricted forced breathing left him apnoeic for 47 and 60 sec., 1 and 1½ min. of diaphragmatic forced breathing produced apnoeic periods of under 10 sec. The sensations of incipient tetany were equally apparent, so it is probable that the magnitude of the voluntary overventilation was comparable in the two pairs of experiments. On two occasions, when the magnitude of the ventilation during forced breathing was measured on J.N.M., it was found to be 76 and 83 l./min. for thoracic and diaphragmatic forced breathing respectively.

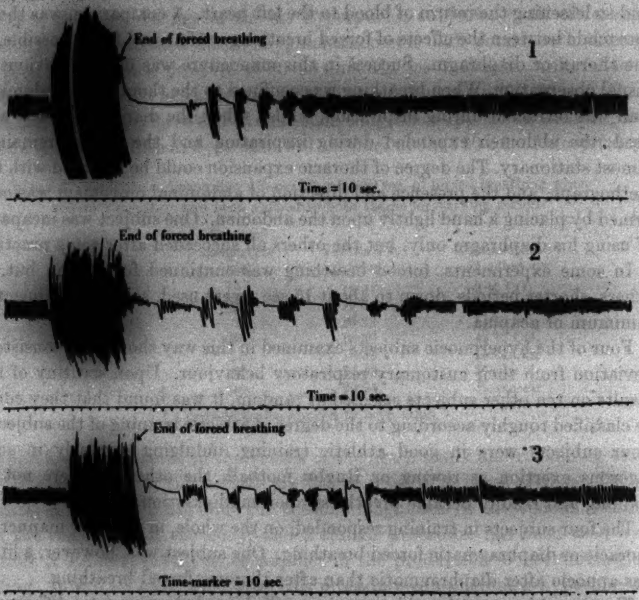

Fig. 4. Subject J.N.M. Stethograph records of 1¼ min. forced breathing. 1, thoracic. 2, diaphragmatic. 3, diaphragmatic, after both thighs had been encircled for ¾ hr. by cuffs inflated to 80–90 mm. Hg. Arrow represents end of forced breathing. Inspiration downwards. No alveolar sample.

In three subjects the respiration rate steadily or abruptly increased during the second half of 1 min. diaphragmatic forced breathing despite efforts to keep it constant. The subjects remarked afterwards that the forced breathing had become much easier, except that they wanted to use the thorax as well as the diaphragm. The appearance was of some reflex stimulus powerfully affecting the respiratory centre, and not confining itself to the diaphragm.

Further recordings were made upon a female student observed in class. She was doing a spell of diaphragmatic forced breathing whilst her partner attempted to measure her blood-pressure fluctuations. After about ½ min. she desisted and started breathing heavily with both thorax and diaphragm, explaining afterwards that 'she couldn't go on any longer, she was so out of breath'. On subsequent repeated examination she always showed severe hyperpnoea after as little as 15 sec. diaphragmatic forced breathing, and only a slight continuance of respiration after thoracic forced breathing. Other subjects agreed that the sensation after diaphragmatic forced breathing was indistinguishable from the dyspnoea of vigorous unaccustomed exertion. The absence of the phenomenon in subjects in athletic training, that is, in subjects whose circulatory system is adapted to pass on large increases in systemic venous return, supports the possibility that this hyperpnoea may be a consequence of increased venous return, presumably due to a rise of pressure at some point in or near to the pulmonary circuit.

In three subjects showing obvious hyperpnoea only after diaphragmatic forced breathing, the experiment was repeated with sphygmomanometer cuffs round the thighs at 80–90 mm. Hg for 10–30 min. before the overbreathing began. In two subjects this abolished the hyperpnoea, whilst in all three there was subjectively no longer the same respiratory distress. One of these tracings is shown in Fig. 4.

With J. N. M., in whom it seemed desirable to exclude any possible psychological effect of expectation of the result, an attempt was made on one occasion voluntarily to remain apnoeic after 75 sec. of vigorous diaphragmatic forced breathing. This became increasingly difficult and, after 9 sec., was no longer possible, and an unusually violent hyperpnoea then ensued and lasted for 25 sec. On two other occasions the subject was nearly asleep on the couch before he started forced breathing, and fell asleep immediately afterwards, having no idea when he awoke what had been his respiratory behaviour; but, although one of these was a thoracic and the other a diaphragmatic spell of forced breathing, the usual apnoea and hyperpnoea occurred.

The subjects examined appear to fall into three groups. In the first group are subjects who are subsequently hyperpnoeic whether they perform forced breathing with the thorax or diaphragm and their hyperpnoea is unaffected by occluding blood in the limbs; they may or may not be in athletic training. The second group is made up of subjects who are in good athletic training and are not hyperpnoeic after thoracic or diaphragmatic forced breathing; the muscles they use during forced breathing do not affect the respiratory sequel. The third group is composed of subjects in poor athletic training. In these subjects, diaphragmatic forced breathing appears to provide a respiratory stimulus which shortens or prevents subsequent apnoea and may even produce hyperpnoea which is prevented by venous congestion of the lower limbs.

The obvious inference is that increased venous return, produced by diaphragmatic forced breathing, can stimulate respiration in subjects in poor athletic training, but that such an increase of venous return is not responsible for the behaviour of the subjects who are regularly hyperpnoeic.

Cortical factors. The cerebral cortex might be implicated in the causation of hyperpnoea after forced breathing in two ways: the act of forced breathing might indirectly affect cortical activity, as by producing cerebral vasoconstriction; or the cortical activity initially responsible for the forced breathing might be a direct cause of the continued hyperpnoea.

The acapnia induced by forced breathing often causes, directly or indirectly, certain abnormalities of cortical activity, such as transitory loss of consciousness, confusion, and the appearance in the electroencephalogram of low-frequency, high-potential delta waves. In the small series of thirteen subjects on whom electroencephalographic records (e.e.g.) were obtained, there was no significant association between the occurrence of delta waves and loss of consciousness after forced breathing; two subjects became confused or unconscious and readily showed delta waves; four showed delta activity but retained perfectly clear consciousness; one very easily lost consciousness after as little as 1 min. of forced breathing but showed no delta activity; and six showed neither delta activity nor impairment of consciousness. It is well known (Brazier, Finesinger & Schwab, 1944; Davis & Wallace, 1942; Engel & Margolin, 1942; Lennox, Gibbs & Gibbs, 1938) that delta activity is more likely to follow forced breathing if the blood sugar is low. The routine e.e.g. were obtained at least 3 hr. after the previous meal. In addition, the effect of forced breathing upon three subjects in whom the blood-sugar concentration had been reduced by the intravenous injection of 1·6–3 units of insulin 15–20 min. previously has been investigated. In every case forced breathing caused both delta activity and loss of consciousness. The protocols of two such experiments are given.

J.N.M. 19. viii. 43. Fasting except for one cup of black coffee. Insulin 2 units intravenously. E.e.g. tracings after 4, 5, 6 and 19 min. showed no abnormality. Unsuccessful attempts to obtain blood sample at 15 min. At 20 min. forced breathing started, with usual instructions for subject to lift index finger to indicate that he heard a call of the time which was given every 15 sec.

Subject responded to '15 sec.', did not respond to '30 sec.', responded to '45 sec.', did not respond again and was apparently unconscious for 4 min. Stopped overbreathing after 82 sec., breathing then became periodic, probably rather more violent than usual, subject showed signs of tetany. After about 4 min. subject asked if forced breathing was still required, and was told 'no'. Breathing continued to be periodic. Blood sample, obtained from superficial vein in arm 30 min. after insulin, 10 min. after start of forced breathing, contained glucose 50 mg./100 c.c.

Intense cutaneous vasoconstriction, subject felt very cold, and this condition continued, with profuse sweating, for about ½ hr. E.e.g., portions of which are reproduced in Fig. 5, showed delta activity with each hyperpnoeic spell, subsiding during the ensuing apnoea.

There was no instrumental record of breathing in this experiment, but the start and finish of each period of apnoea were marked on the e.e.g. tracing by

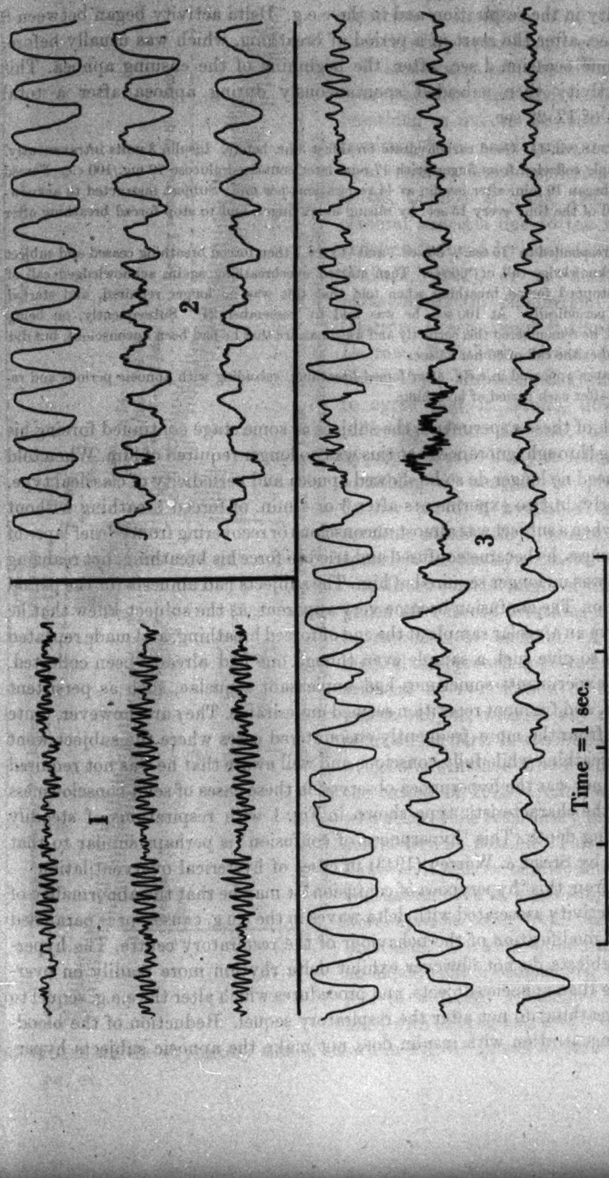

Fig. 5. Subject J. N. M. Electroencephalographic tracings. 1, normal fasting; 2 and 3, portions of tracing after insulin injection and forced breathing, when subject was breathing periodically and apparently unconscious. 2, while subject was breathing. 3, during apnoea, showing disappearance of delta rhythm. Each set shows, from above downwards, tracings from frontal, parietal and occipital electrodes.

hand, which allowed an approximate calculation of the time relations of the periodicity in the respiration and in the e.e.g. Delta activity began between 8 and 18 sec. after the start of a period of breathing, which was usually before, but on one occasion 1 sec. after, the beginning of the ensuing apnoea. This delta activity then subsided spontaneously during apnoea after a total duration of 12–22 sec.

M. W. G. 18. vii. 44. Good carbohydrate breakfast 3 hr. before. Insulin 3 units intravenously. Blood sample collected from finger-prick 17 min. later contained glucose 70 mg./100 c.c. Forced breathing began 19 min. after insulin at 44 respirations per min. Subject instructed to acknowledge a call of the time every 15 sec. by raising index finger, and to stop forced breathing after 1 min.

Subject responded to '15 sec.', '30 sec.', and '45 sec.', then forced breathing ceased and subject did not acknowledge call of '60 sec.' Then started overbreathing again, acknowledged call of '75 sec.', stopped forced breathing when told that this was no longer required, and started breathing periodically. At 100 sec. he was told to 'remember 21'. Subsequently, on being questioned, he remembered this correctly and was unaware that he had been unconscious, but did not remember the call of 60 nor 75 sec.

Delta waves appeared in e.e.g. after forced breathing, subsiding with apnoeic periods and reappearing after each period of breathing.

In each of these experiments the subject at some stage continued forcing his breathing through ignorance that this was no longer required of him. When told that he need no longer do so he showed apnoea and periodicity of classical type.

Similarly, in two experiments after 3 or 4 min. of forced breathing without insulin, when a subject was almost unconscious or recovering from a brief lapse of consciousness, he became confused and tried to force his breathing, not realizing that this was no longer required of him. The subjects had amnesia for the period in question. The confusion became very apparent, as the subject knew that he had to give an alveolar sample at the end of forced breathing, and made repeated attempts to give such a sample even though one had already been collected.

These experiments sometimes had unpleasant sequelae, such as persistent headache, and frequent repetition seemed undesirable. They are, however, quite different from the more frequently encountered cases where the subject went on overbreathing while fully conscious and well aware that he was not required to do so; nor was the hyperpnoea observed in these cases of semi-consciousness ever of the characteristic type shown in Fig. 1 with respirations of steadily diminishing depth. This 'hyperpnoea of confusion' is perhaps similar to that observed by Stead & Warren (1943) in cases of hysterical overventilation.

Apart from this 'hyperpnoea of confusion' it may be that the abnormality of cortical activity associated with delta waves in the e.e.g. causes, or is paralleled by, some modification of the behaviour of the respiratory centre. The hyperpnoeic subjects do not however exhibit delta rhythm more readily on overbreathing than apnoeic subjects, and procedures which alter the e.e.g. sequel to forced breathing do not alter the respiratory sequel. Reduction of the blood-sugar concentration with insulin does not make the apnoeic subjects hyperpnoeic after forced breathing except when they are confused, nor does its elevation by ingestion of 50 g. glucose make the hyperpnoeic subjects apnoeic.

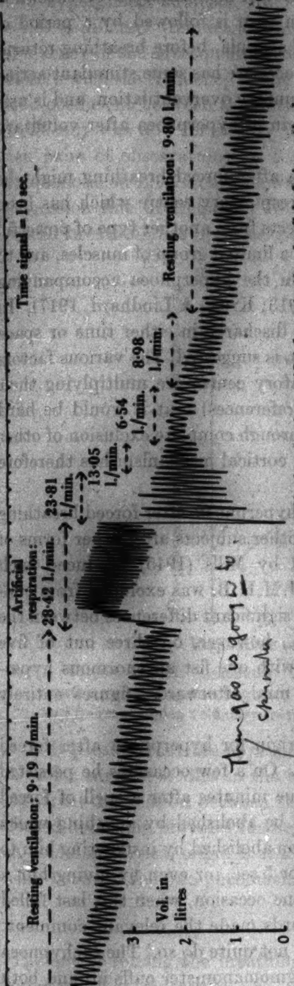

Fig. 6. Subject J.N.M. Spirometer tracing before, during, and after 1 min. artificial respiration by Schafer's method. Inspiration downwards. Calculated ventilation l./min. S.T.P. dry over successive periods inserted on tracing.

Then gas is going to chamber

There seems as yet to be no final agreement whether delta activity after forced breathing is due to cerebral acapnia resulting from inadequate or absent compensatory vasoconstriction (Gibbs, Gibbs, Lennox & Nims, 1942), or to cerebral anaemia due to the vasoconstriction induced by acapnia (Davis & Wallace, 1942), or to decrease of parasympathetic cholinergic activity (Darrow, Green, Davis & Garol, 1944; Darrow, McCulloch, Green, Davis & Garol, 1944). However, all workers seem to agree that it results, directly or indirectly, from acapnia; and since hyperpnoea is as likely to follow forced breathing if acapnia is prevented, further proof is available that this cortical abnormality cannot be responsible for the hyperpnoea.

A direct cortical origin of the hyperpnoea after forced breathing is difficult to establish or exclude. A possible method of approach appeared to be the artificial overventilation of a hyperpnoeic subject, without any muscular effort on his part. It was found possible by Schafer's method to overventilate, to the extent of 20–30 l./min., a subject who relaxed his respiratory muscles as completely as possible. This resulted, however, in a period of involuntary overventilation even in some subjects who were apnoeic after voluntary forced breathing. A tracing obtained in such an experiment by connecting the subject to a Krogh spirometer initially filled with oxygen and with a soda-lime absorbent on the expiratory side is shown in Fig. 6. It will be seen that the artificial overventilation is followed by a period of involuntary overventilation performed by the subject, and that this in turn is followed by a period of hypopnoea, presumably the consequence of acapnia, before breathing returns to normal. Clearly, in some subjects, the procedure has some stimulant action on respiration which is not produced by voluntary overventilation, and is useless for investigating a possible cortical origin of hyperpnoea after voluntary forced breathing.

A primary cortical origin for hyperpnoea after forced breathing might be analagous to the increased activity of the respiratory centre which has been supposed (Mills, 1945) to result in some subjects from another type of powerful cortical discharge, a brief intense effort with a limited group of muscles, and to the supposed participation of the cortex in the hyperpnoea accompanying more severe exertion (Krogh & Lindhard, 1913; Krogh & Lindhard, 1917). In all these circumstances a spread of cortical discharge in either time or space beyond its deliberately intended distribution is suggested. The various factors alleged to affect the activity of the respiratory centre are multiplying their number so rapidly (see Comroe (1944) for references) that it would be hard confidently to assert a cortical mechanism through complete exclusion of other possibilities. Direct evidence in favour of a cortical mechanism has therefore been sought.

It was hoped that those subjects who are hyperpnoeic after forced breathing might show hyperpnoea more readily than other subjects after other forms of effort. Unfortunately, the technique used by Mills (1945) produced such variable results in any one subject that, if J. M. H. B. was excluded from consideration, it was impossible to establish any significant differences between the behaviour of different subjects. J. M. H. B., however, on three out of five occasions produced after a 10 sec. squeeze with one fist an enormous hyperpnoea, breathing at 16–23 l./min. for 2–3 min. afterwards, figures entirely outside the range shown by other subjects.

Further evidence suggesting a cortical origin for hyperpnoea after forced breathing in this subject was also obtained. On a few occasions he persisted with rather rapid shallow breathing for some minutes after a spell of forced breathing, and when this occurred it could be abolished by anything which sufficiently diverted his attention. It has been abolished by instructing him to take a deep breath, or to hold his breath for 5 sec., or even by giving him a simple problem in mental arithmetic. On one occasion, when this last failed to stop the tachypnoea, the subject afterwards made the relevant comment: 'I thought it was going to stop it, but it did not quite do so.' The tachypnoea was entirely unaffected by inflation of sphygmomanometer cuffs around both thighs at a pressure of about 80 mm. Hg. A cortical origin of the hyperpnoea seems to be the most likely explanation.

A further observation suggesting a cortical origin was that, in three out of five hyperpnoeic subjects, by whom forced breathing was performed at a series of different frequencies varying from 12 to 60 respirations per min., the frequency of the subsequent involuntary hyperpnoea often corresponded fairly closely with the frequency of the previous forced breathing. Thus, the correlation coefficient between the initial voluntary and the subsequent involuntary frequency was 0·71 for twenty pairs of observations on J.E.C., and 0·90 for ten pairs of observations on L.D.H., both highly significant figures. These frequencies on L.D.H. are shown in graphic form in Fig. 7, and the calculated regression line of frequency after forced breathing upon frequency during forced breathing has been inserted.

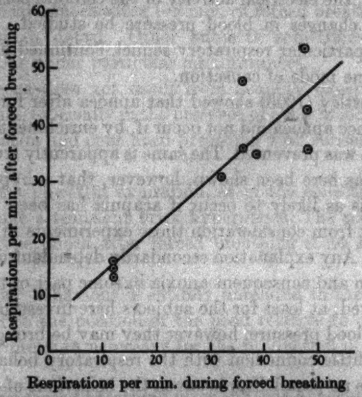

Fig. 7. Subject L.D.H. Respiratory frequency during involuntary hyperpnoea after forced breathing plotted against frequency during forced breathing, with regression line of involuntary frequency upon voluntary frequency inserted.

It is possible that the hyperpnoea, provoked in some subjects by giving an alveolar sample, is due to the effort involved. If this is the case, then the apparent association between this phenomenon and a hyperpnoeic sequel to forced breathing provides further evidence for the cortical origin of the latter. A common cortical origin is at least the most easily envisaged causal relationship between the two phenomena.

DISCUSSION

The act of forcing the breathing has numerous direct and secondary effects upon the cardiovascular and nervous systems, and these react upon one another. The primary phenomenon may be considered as a powerful cortical discharge whose precise distribution is entirely unknown, but which greatly increases the respiratory movements. The consequent overventilation of the lungs alters the chemical composition of the blood; and the muscular movements involved in breathing produce large rhythmic fluctuations in intrathoracic and intra-abdominal pressures which mechanically affect the circulation of the blood. The chemical changes may directly affect the activity of different levels of the central nervous system, and also, by direct or reflex action on the heart and vascular calibre, produce alterations in the circulation; alterations in the circulation, produced directly or indirectly, may alter blood flow through parts of the central nervous system and hence produce chemical changes there. It is not surprising, therefore, that the result of even the most carefully standardized overbreathing procedure is very variable, whether the respiratory sequel, the electrical activity of the cerebral cortex, the changes in consciousness, or changes in blood pressure be studied. Equally, it is not surprising that a particular respiratory sequel, continued hyperpnoea, should have more than one mode of causation.

Haldane & Priestley (1905) showed that apnoea after forced breathing was due to acapnia, since apnoea did not occur if, by enrichment of the inspired air with CO_2, acapnia was prevented. The same is apparently true of delta activity in the e.e.g. It has here been shown, however, that a hyperpnoeic sequel to forced breathing is as likely to occur if acapnia has been minimized or prevented, if we omit from consideration those experiments in which the subject became confused. Any explanation secondarily dependent upon acapnia, such as vasoconstriction and consequent anoxia in some part of the carotid supply, is likewise negatived, at least for the subjects here investigated.

Alterations in blood pressure, however they may be brought about, seem to have remarkably little connexion with the respiratory behaviour after forced breathing. There is no parallelism between the behaviour of the blood pressure and of the breathing, and procedures designed to exaggerate or minimize the effects of a falling blood pressure upon cerebral blood flow do not modify the respiratory sequel to forced breathing. Forced breathing can certainly reduce the blood pressure, and the pressure may not return to normal rapidly afterwards, but any such reduction does not appear to be responsible for respiratory stimulation.

An increased venous return, induced particularly by diaphragmatic overventilation, is sometimes a cause of continued hyperpnoea. The hyperpnoea is decreased or absent if the venous return is limited by occlusion of blood in the lower limbs. Speculation upon the possible receptors is premature in the present state of our knowledge of the different possible types of vagal afferent fibres from pulmonary vessels and of their central connexions and reflex effects.

Some subjects, however, are persistently hyperpnoeic after forced breathing whatever measures are taken to exclude, diminish or increase the possible modes of stimulation of the respiratory centre. The hyperpnoea is independent of acapnia and the giving of an alveolar sample, and quite uncorrelated with blood-pressure changes; it is undiminished if the venous return is restricted by occlusion of blood in the lower limbs; it is uncorrelated with modifications of electrical activity of the cerebral cortex induced by forced breathing. It is therefore suggested, in part by exclusion and in part through some positive lines of evidence, that it results directly from that same cortical activity which is initially responsible for the forced breathing.

SUMMARY

1. Ten subjects, out of forty examined, regularly showed hyperpnoea instead of the classical apnoea after 1–2 min. of forced breathing. Six of these have been studied in detail. Other subjects sometimes showed similar respiratory behaviour under particular circumstances.

2. This hyperpnoea does not result from acapnia, excepting in a few instances when acapnia, especially in the presence of a low blood-sugar concentration, leads to confusion and continued breathing with subsequent amnesia for the event.

3. The giving of a powerful expiration, as in the collection of an alveolar sample, may provoke a transient brisk hyperpnoea. The six subjects investigated still show hyperpnoea after forced breathing if no alveolar sample is given.

4. The respiratory behaviour is entirely unrelated to the behaviour of the blood pressure and to the position of the head relative to the heart, nor is a reduction of blood pressure necessary for the production of hyperpnoea after forced breathing.

5. Diaphragmatic forced breathing often induces hyperpnoea in subjects in whom thoracic forced breathing has no such effect. Reasons are given for supposing that this is a reflex consequence of increased venous return.

6. Some observations are made on the changes in cortical potentials induced by forced breathing. These are entirely unrelated to the respiratory behaviour.

7. In some subjects involuntary hyperpnoea still follows forced breathing if all known reflex causes are as far as possible excluded. The possibility is considered that this is due directly to the cortical activity which produces the forced breathing.

My thanks are due to all those who have acted as subjects, and to Mr T. J. Meadows for technical assistance. Electroencephalographic recordings were performed by Miss D. J. Dow, of the Nuffield Department of Surgery, to whom also my thanks are due.

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THE SITE OF PRESSOR ACTION OF THE BASE A, CLOSELY ALLIED TO *l*-NICOTINE, PRESENT IN URINE

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(*Received* 6 *November* 1945)

The extraction of this base from human urine, and its similarity to *l*-nicotine has been described (Lockett, 1944). The base has also been found in dog urine, where it was shown to be of endogenous origin (Lockett, 1945). Certain discrepancies arose if the original form of the base were considered to be *l*-nicotine; these could be explained on the hypothesis that the base was excreted in a form differing from *l*-nicotine, but readily converted to *l*-nicotine: this theoretical form was termed base A (Lockett, 1944). The base A, when freshly isolated either as the oxalate or the picrate by the lead precipitation method, is pharmacologically more active than *l*-nicotine, and recrystallization does not lessen the activity. By the simple procedure of subjecting salts of base A to a high vacuum the pressor activity was reduced to that of *l*-nicotine: the analytical figures then corresponded very closely with the calculated results for nicotine picrate. It is to be shown below that not only is the pressor activity of the base A reduced by subjection of its salts to a high vacuum, but the site of its action in the animal is completely changed. Moreover, *l*-nicotine when added to urine in moderate amount was constantly precipitated by basic lead acetate and readily adsorbed on lead sulphide, but the base A was not. Finally, some evidence was obtained of the presence of a carbonyl group in the base A. These results all suggested that the original form, A, in which the base was excreted differed from *l*-nicotine.

The experiments recorded below confirm this view. They show that the base A acts on or through the region of the corpus striatum, thalamus, and fornix in the brain; it is only after treatment in a high vacuum that the base has any peripheral nicotine-like action.

A semicarbazone has been prepared from the base A.

METHODS

Preparation of solutions

The oxalate of the base A was prepared by the lead precipitation method as previously described (Lockett, 1944). It was recrystallized from absolute alcohol, and dried in air. M.p. 113° C. Analysis (Weiler): C, 45·6%; H, 5·4%; N, 6·3%.

Solutions of adrenaline tartrate were prepared from the crystalline salt immediately before use.

Pharmacological preparations

Anaesthesia was induced in cats with chloroform and ether (1 : 2), a cannula was tied into the right femoral vein and a solution of chloralose (1% in 0·9% NaCl) infused to a dose of 0·08 g./kg. body weight. Solutions for testing were warmed, and were given through the same cannula, and were washed in with 2 c.c. of warm 0·9% NaCl. A tracheal cannula was inserted, and the blood pressure was recorded from the left common carotid artery, clotting in the cannula being prevented by filling it with a solution of heparin in 0·9% NaCl (1 mg./c.c.).

Decerebration. The right common carotid artery of the cat was tied; the left temporal muscle was reflected; the cranium trephined, and the dura incised. The cerebral peduncles were divided by means of a blunt blade passed through the opening and moved in an anteroventral direction. The temporal muscle was replaced, and the skin closed by suture.

In exposure of the brain, both temporal muscles were reflected, multiple trephine holes made, and the vault of the cranium removed. The dura was opened on either side of the mid-line, the longitudinal sinus excised between ligatures, and the dura reflected.

Acute sympathectomy. Artificial respiration was applied; the sternum was divided in the mid-line, and one side of the thoracic wall was retracted. Starting from the stellate ganglion, all the sympathetic ganglia on each side were freed by division of the rami, the roots of the splanchnic nerves were cut, and the whole sympathetic chain removed down to the crus of the diaphragm. The chest was then closed. The abdomen was next opened, and the abdominal sympathetic chains were isolated by division of the ganglionic rami; the chains were removed to the point at which they dipped into the pelvis, and the abdomen was closed.

RESULTS

The site of pressor action of base A

It was noted on two occasions that after ligature of both common carotid arteries, cats, under chloralose anaesthesia, failed to respond to the intravenous injection of the base A. The effect of decerebration was therefore investigated, and the action of the base A compared with that of adrenaline. The results are summarized in Table 1.

TABLE 1. The site of pressor action of the base A

			Blood pressure (mm. Hg)		
Cat	Conditions	Intravenous injection	Base-line	Rise	Fig.
1	Under chloralose anaesthesia	22 μg. oxalate base A	143	49	1*a*
		10 μg. adrenaline tartrate	144	52	1*b*
	Decerebrate	22 μg. oxalate base A	74	4	1*c*
		10 μg. adrenaline tartrate	78	128	1*d*
2	Chloralose, brain exposed	33 μg. oxalate base A	101	78	2*a*
	After removal of the cerebral hemispheres and corpus callosum	33 μg. oxalate base A	84	124	2*b*
	After removal of thalamus, fornix, and corpus striatum	99 μg. oxalate base A	78	5	2*c*
		20 μg. adrenaline tartrate	79	68	2*d*
3	Under chloralose anaesthesia	20 μg. adrenaline tartrate	151	30	
		44 μg. oxalate base A	146	28	
	After acute sympathectomy	10 μg. adrenaline tartrate	49	104	
		66 μg. oxalate base A	54	3	

Cat 1. Weight 2·5 kg. Under simple chloralose anaesthesia, the rise in blood pressure following the intravenous injection of 22 μg. of the oxalate of the base A was slightly less than after 10 μg. of adrenaline tartrate (Fig. 1*a*, *b*). After decerebration there was an increased response to 10 μg. of adrenaline, but the response to 22 μg. of base A oxalate was abolished (Fig. 1*c*, *d*). After the brain had been fixed it was examined. The section had passed through the cerebral peduncles, entering the dorsal surface just anterior to the anterior colliculi, and had emerged between the corpora mamillaria and the anterior edge of the pons.

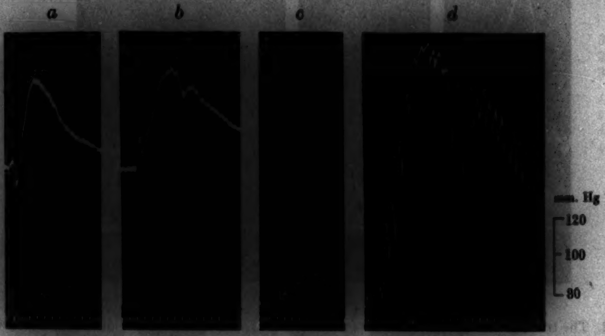


Fig. 1. The tracings show the responses of the arterial blood pressure to the intravenous injection of the following salts: (*a*) and (*c*), 22 μg. of the oxalate of the base A; (*b*) and (*d*), 10 μg. of adrenaline tartrate. Between (*b*) and (*c*) decerebration was performed. Time marker, 10 sec.

The pressor action of the base A is therefore produced on or through some part of the brain anterior to the mesencephalon. The exact site of action was further localized by removing successive parts of the brain.

Cat 2. Weight 2·6 kg. Under chloralose anaesthesia the vault of the cranium was removed, and the brain exposed. The pressor response to 33 μg. of oxalate of the base A was recorded (Fig. 2*a*). The cerebral hemispheres and the corpus callosum were removed, and the response to the same dose again recorded (Fig. 2*b*). The thalamus, corpus striatum and fornix were then removed; there was now no response to the injection of 99 μg. of oxalate of the base A, but the cat responded to 20 μg. of adrenaline tartrate (Fig. 2*c*, *d*).

The base A therefore acts on or through the region of the corpus striatum, thalamus, and fornix, and the following experiment shows that the sympathetic system is also involved.

Cat 3. Weight 2·9 kg. Under chloralose anaesthesia 20 μg. of adrenaline tartrate produced a rise in blood pressure of 30 mm. Hg. from a base-line of 151 mm. Hg. Under the same conditions 44 μg. of the oxalate of the base A gave a rise of 28 mm. Hg from a base-line of 146 mm. Hg. After total acute sympathectomy, whereas only 10 μg. of adrenaline tartrate still produced a large rise in blood pressure (104 mm. Hg from a base-line of 49 mm. Hg), an increased dose (66 μg.) of the oxalate of base A was without effect (base-line 54 mm. Hg).

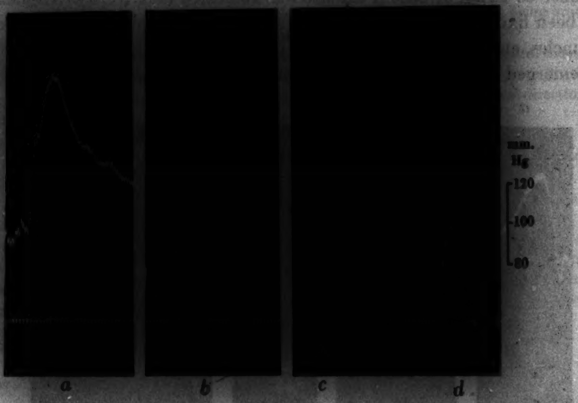


Fig. 2. The tracings show the responses of the arterial blood pressure to the intravenous injection of the following salts: (*a*) 33 μg. of the oxalate of the base A after exposure of the brain; (*b*) the same dose after the removal of the cerebral hemispheres and corpus callosum; (*c*) 99 μg. of this oxalate after the removal of the thalamus, fornix, and corpus striatum; (*d*) 20 μg. of adrenaline tartrate. Time marker, 5 sec.

The pressor action of the base A is therefore produced in the region of the corpus striatum, thalamus and fornix, and becomes effective through the sympathetic nervous system.

The change in site of pressor action when the base A is converted to l-*nicotine*

Unfortunately, owing to the wartime shortage of cats, these investigations were much curtailed, but in view of the experiments reported in the previous section, the results recorded below are of significance, and publication is therefore no longer delayed.

The base A appears to be more active than *l*-nicotine (Lockett, 1944). The following single experiment on a cat weighing 3·0 kg., and under chloralose anaesthesia, is described in Fig. 11 of the former paper, and in Table 2, as cat 4, in this paper, and makes this point quite clear.

Under simple chloralose anaesthesia, as little as 66 μg. (cf. 87 μg. nicotine picrate) of the picrate, and 24 μg. (cf. 63 μg. nicotine oxalate) of the oxalate of the base were found to produce the same effect as 71 μg. of *l*-nicotine-*dl*-acid tartrate, $2H_2O$. The figures in brackets are those calculated for the corresponding salts of *l*-nicotine, the dose given being that which would be expected to produce a pressor response equal to that of 71 μg. of *l*-nicotine acid tartrate.

TABLE 2. The change in site of action when the base A is converted to *l*-nicotine

Cat	Conditions	Intravenous injection	Blood pressure (mm. Hg) Base-line	Blood pressure (mm. Hg) Rise	Fig.
4	Chloralose anaesthesia	66 μg. picrate base A	155	44	
		24 μg. oxalate base A	156	46	
		71 μg. tartrate *l*-nicotine	149	45	
	Decerebrate	144 μg. oxalate base A	128	Nil	3*a*
		264 μg. picrate base A	126	26	3*b*
		284 μg. tartrate *l*-nicotine	126	146	3*c*
5	Decerebrate under chloralose	95 μg. picrate *l*-nicotine	92	52	4*a*
		90 μg. } vacuum treated	92	48	4*b*
		60 μg. } picrate base A	100	32	4*c*
		63 μg. picrate *l*-nicotine	98	34	4*d*

Fig. 3. The tracings show the responses of the arterial blood pressure to the intravenous injection of the following salts: (*a*) 71 μg. of *l*-nicotine tartrate; (*b*) 24 μg. of the oxalate of base A; (*c*) 144 μg. of this oxalate; (*d*) 164 μg. of base A picrate; (*e*) 284 μg. of *l*-nicotine tartrate. Decerebration performed between *b* and *c*. Time marker, 5 sec.

Whereas the injected dose of the oxalate contained 1·5 μg. of basic nitrogen, that of the picrate contained 3·0 μg. The oxalate therefore has greater pressor activity than the picrate; this is discussed below. After these results had been obtained, the cat was decerebrated; the section passed through the peduncles, just anterior to the pons. The salts were then again tested, and the results are shown in Table 2 and in Fig. 3. Six times the original dose of the oxalate of base A was now without pressor effect; and whereas the pressor response to four times the original dose of the picrate of base A was very small, that to four times the original dose of *l*-nicotine was supramaximal.

When the picrate had been dried in a high vacuum at room temperature, the analytical figures agreed very closely with those calculated for nicotine picrate, the melting points were similar, and the mixed melting points satisfactory. Calculated: C, 42·6%; H, 3·1%; N, 18·1%. Found: C, 42·58%; H, 3·3%; N, 18·06%.

Comparison of pressor activity was therefore made again with *l*-nicotine. In a cat weighing 1·6 kg. and under chloralose anaesthesia (Lockett, 1944, Table 8 and Fig. 12), the pressor activity of *l*-nicotine picrate was found to equate with that of the vacuum-dried picrate of the base A. These two picrates were therefore tested for pressor activity on a decerebrate cat, weighing 1·8 kg., in which the section passed through the cerebral peduncles just anterior to the pons. The results are shown in Fig. 4 and in Table 2, cat 5. The rise in blood pressure following the intravenous injection of 63 μg. of *l*-nicotine picrate was now found to equate with that following 60 μg. of the treated picrate of the base A.

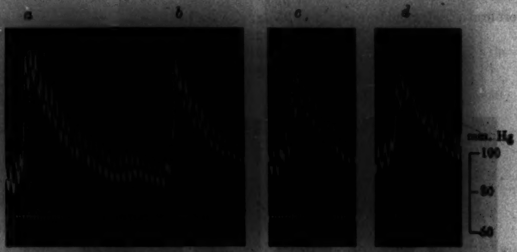


Fig. 4. The tracings show the responses of the arterial blood pressure of a decerebrate cat to the injection of the following salts: (*a*) 93·5 μg. and (*c*) 63 μg. of *l*-nicotine picrate; (*b*) 90 μg. and (*d*) 60 μg. of the vacuum-treated picrate of the base A. Time marker, 5 sec.

Not only is the pressor activity of the picrate of the base A reduced to equal that of *l*-nicotine picrate by the simple procedure of drying the salt in a high vacuum, but the site of pressor action has also changed. Initially it was central in type, the peripheral action of nicotine being absent in the highly active oxalate, and very greatly reduced in the less active picrate. After drying in a high vacuum, the picrate of the base A has the same pressor activity as *l*-nicotine; its action is no longer central, but like that of *l*-nicotine is peripheral. Moreover the analytical figures for the vacuum-dried base A accord closely with those calculated for nicotine.

The very active oxalate of the base A had a purely central action, and a pressor activity two and a half times as great as that calculated for *l*-nicotine. Before vacuum treatment, the picrate had both central base A action and peripheral nicotine-like action, and a pressor activity one and a half times as great as that calculated for *l*-nicotine. By treatment of this picrate in a high vacuum, all central base A action was lost, full peripheral nicotine activity was acquired, and the total pressor action was reduced to that of *l*-nicotine. This picrate must therefore have undergone partial alteration from base A to *l*-nicotine before the first testing. Although the oxalate described above was not tested after vacuum treatment, from a very highly active oxalate of the base A, having pure central and no peripheral action, a picrate was prepared which after treatment in a high vacuum had only peripheral nicotine action, and which equated with, and analysed as, *l*-nicotine picrate.

There is but one possible conclusion, the base A differs from *l*-nicotine by some additional volatile group. The ultra-violet absorption spectrum of the base A very closely resembles that of *l*-nicotine, and intensifies in acid solution.

The isolation of a semicarbazone from the base A

Evidence that the base A may react with carbonyl reagents was given in a previous paper (Lockett, 1944, § 3). Attempts to isolate the oxime and phenyl hydrazone failed; a bisulphite compound was obtained in very small amount. A semicarbazone was obtained in crystalline form from 15 mg. of pure oxalate of the base A.

Method. 15 mg. of pure anhydrous oxalate of the base A were dissolved in 1 c.c. of water, made alkaline with 0·1 c.c. of 2·5N-NaOH, and three times extracted with ether; the combined ether extracts were shaken with 2 c.c. of 0·175N-HCl, and the aqueous solution was warmed in a water-bath at 80° C. until all traces of ether were removed. After cooling and neutralizing with caustic soda, a knife-point of semicarbazide hydrochloride and excess sodium acetate were added. The whole was heated on a boiling water bath for 2 hr. and cooled. A small amount of white crystalline solid separated; this was washed with water and ice-cold alcohol, and was dried over P_2O_5. Yield 1·9 mg., m.p. 181° C. The nitrogen content was determined. 1·71 mg. yielded 0·578 c.c. N at 758/23. N, 38·8%. A control experiment using 15·6 mg. *l*-nicotine oxalate yielded no precipitate.

The oxalates of the base A have already been discussed, and an abnormal oxalate of *l*-nicotine described. The theory was advanced that the oxalate described for the base A, corresponding to a formula of

$$C_{16-17}H_{20-21}N_2O_{11-12}(COOH)_5,$$

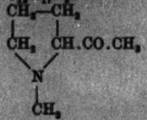
the oxalic being determined by Ca precipitation followed by titration with acid permanganate, might be a mixture of typical and atypical oxalates. The C, H and O values, which were higher than those expected for *l*-nicotine, were shown not to be due to water or alcohol of crystallization, and may therefore be taken to belong to a side-chain. In Table 3 are shown the % N content of semicarbazones from possible groups.

Discussion of the structural formula of the base A

The main C structure of the base A has been identified as *l*-nicotine, from which the base A is shown to differ by an additional volatile group. The presence of a carbonyl group in the base A has been indicated, and from the base A a semicarbazone has been isolated; it can only be derived from a side chain whose nature and position has yet to be discussed.

The great solubility of the base A in ether would argue against the theory of a carboxylic ketonic acid of N-Me pyrrolidine, or of pyridine, or of a betaine structure such as is present in stachydrine. The solubilities are consistent with a ketonic ester or an aliphatic ketonic side-chain. The base A shows reduction of pressor activity by one third to one half on boiling with dilute acid or alkali for 1 hr., or by standing with 7·5 N-NaOH for 48 hr. It remained unchanged in 11 N-H_2SO_4 for 48 hr., and *l*-nicotine was found to undergo no alteration under these conditions, racemization therefore not occurring: the reduction of activity in the case of the base A may therefore be associated with its conversion to *l*-nicotine. Such findings would be compatible with an acetoacetic ester side-chain, but this is made unlikely since the base A, tested in solution as the hydrochloride, gave no violet colour with ferric chloride. The production of an orange colour with α-naphthol and alkali again indicates the presence of a carbonyl group. The theory of a ketonic side-chain is suggested by the failure of the base to benzoylate by the Schotten-Baumann method, and the ability of the base to react as if a free carbonyl group were present. A side-chain such as —$CH_3.CO.CH_3$, or acetone, as in hygrine, would not be expected to be readily volatile. Since the absorption spectrum of base A is almost identical with that of *l*-nicotine, and intensifies in acid solution, the N of the pyridine ring must be free, and a ketonic side-chain attached to the pyridine nucleus might be expected to produce some shift in the position of the band.

TABLE 3. The % N and melting-point of semicarbazones from possible groups of a side-chain

Group	m.p. ° C.	% N
$CH_3.CHO$	162	42
$CH_3.CO.CH_3$	187	36·5
$CH_3.CO.COOH$		29
$NH_2.CO.NH.NH.CO.NH_2$	245	51
$C_{13}H_{17}N_3O$		22·5
		32·5

The possible analogy with lobeline was pointed out to me by Dr Ing. The lobeline side-chain contains a secondary alcoholic group which is split off as acetophene when lobeline is heated in aqueous solution, or dry and above its melting point (Weiland, 1921, 1925).

The ultraviolet absorption spectrum of the base A would suggest that any ketonic side-chain must be attached to the pyrrolidine ring. When some of the known alkaloids of pyrrolidine are considered: stachydrine, betonicine and turicine, hygrine and cusk hygrine, cocaine, nicotine and *nor*nicotine, substitution in the α-position and methylation of the nitrogen is the rule in general. Stachydrine, betonicine, and turicine, with betaine structure are pharmacologically inert, but the methyl ester of hygrinic acid to which stachydrine isomerizes on distillation is a central stimulant and convulsant (Trier, 1910). Cocaine has marked action as a central stimulant. Of these alkaloids only nicotine produces characteristic ear twitching in a cat; no ear twitching is produced by the original base A in supramaximal doses in cats in which moderate doses of *l*-nicotine produce energetic ear activity.

SUMMARY

1. The base A acts on the region of the corpus striatum and thalamus, producing its pressor action through the sympathetic nervous system.

2. When the base A is converted to *l*-nicotine the central action is changed to peripheral action.

3. A semicarbazone has been isolated from the base A. The structural formula is discussed.

I would like to express my thanks to Dr E. B. Verney who performed all the operative procedures described in the first section of this paper. All analyses were done by Dr G. Weiler, Oxford.

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THE APPEARANCE OF A BASE, DETECTED BY A COLOUR TEST, IN THE URINE OF BITCHES HAVING RENAL ISCHAEMIA

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In this paper an account is given of the appearance in the urine of a new base within 10 min. of the production of renal ischaemia. This base, detected by a colour test, is not present in normal dog or human urine. In two bitches in which the ureters had been exteriorized, and a single kidney had been rendered ischaemic, the base was found to be excreted mainly by the normal kidney. Since the colour test is given by saline extracts of normal renal cortex, but by none of the sixteen other tissue extracts that have been examined, it would appear probable that the base is liberated by the ischaemic kidney into the blood stream. In confirmation of this, the colour test is given by the blood of bitches during experimental renal hypertension, but not by the blood of these same bitches before the production of renal ischaemia. Finally an account is given of two dogs which were followed throughout two periods of renal hypertension, observations being made on the blood pressure, the presence or absence of this colour test in the blood and urine, and of factors influencing the excretion of the compound or compounds responsible for colour production; to facilitate description, such compounds are referred to as x-positive base.

METHODS

The production of renal ischaemia by constriction of the renal artery

Continuous compression was brought about by the implantation of small silver clips of known diameter around the renal artery, by the method described by Verney & Vogt (1938). When intermittent renal ischaemia was required, use was made of the screw operated compression unit described by Verney & Vogt (1943), or the fluid compression unit of Rydin & Verney (1938). The technique of implantation has been given by these authors.

Exteriorization of ureters

Ureters were extended to the surface by the method of Klisiecki, Pickford, Rothschild & Verney (1933).

Determination of blood pressure

In short-term experiments the dog, which had received preliminary training, lay at rest on a warmed table, and a continuous record of the femoral arterial pressure was made under local anaesthesia, according to the aseptic technique described by Verney & Vogt (1938), clotting in the cannula being prevented by the short infusion of heparin solution. In long-term experiments the dogs were prepared for blood-pressure measurements by the method of the carotid loop with denervated sinus (Verney & Vogt, 1938). Blood-pressure readings were taken at the same time every evening, approximately 1½–2½ hr. after the dog had been fed.

Collection of blood and urine samples

Urine. In the short-term experiments, either a self-retaining catheter was inserted into the bladder, or extensions were applied to the ureteric tubes, each with full aseptic precautions, and the urine was collected over measured periods. For the determination of the output of the base per hour in the long-term experiments, aseptic catheterization was carried out at the same time each morning, and the bladder emptied; after a measured time interval of 1–1½ hr. the animal was again catheterized and the bladder contents collected. Metabolism cages were used when the total output for the day was required.

Blood. From 10 to 14 c.c. of blood were withdrawn aseptically from the malleolar vein into a syringe containing 1 mg. of heparin in 4 c.c. of 0·9% NaCl.

Estimations

Blood urea. These determinations were made by MacLean's modification (1927) of Van Slyke's aeration method.

x-positive base in urine. The amount of *x*-positive base in urine was estimated by a non-specific colorimetric extraction method adapted from that used by Richter (1938) in the estimation of ephedrine in urine. This author states that the method is generally applicable to all higher amines and alkaloids that are extracted from urine by organic solvents. He demonstrated the absence of interfering compounds in normal urine, and this has been confirmed on 140 different samples of normal dog and human urine. Amounts of *x*-positive base colorimetrically equivalent to 5 μg. of ephedrine can be detected; amounts equivalent to 50 μg. can be estimated with an error of 20%, and at a colorimetric equivalent of 100 μg. of ephedrine consistent readings are obtained. The colour produced by ephedrine cannot be distinguished from that of *x*-positive base.

Urine samples were treated as follows: If the total volume of urine for the given period were less than 10 c.c. it was diluted to 10 c.c. with water; if the volume exceeded 10 c.c., 10 c.c. only were taken; 2 c.c. of 10% NaOH were added, and 3 c.c. of purified toluene, in an extraction funnel. Extraction was continued for 5 min. and the fluid was then centrifuged for 15 min. 1·5 c.c. of the upper toluene layer were withdrawn by pipette; 1·5 c.c. of chloroform and 0·1 c.c. of 2% picric acid in chloroform were added, and the solution was compared with the standard in a colorimeter. For the range of concentration of *x*-positive base occurring in urine after the production of renal ischaemia, extraction by toluene from alkaline solution under the given conditions was not complete; $\frac{1}{11}$ of the colorimetric equivalent of the first extraction was obtained by a second extraction. In dilution experiments the amount extracted remained proportional to the degree of dilution. Duplicate extractions gave corresponding results throughout.

The ephedrine standard. 0·01 g. of ephedrine hydrochloride in 50 c.c. of water plus 10 c.c. of 10% NaOH were shaken for 5 min. with 50 c.c. of toluene and then centrifuged; the toluene layer separated and was again centrifuged. The upper 30 c.c. were withdrawn by dropping pipette and stored in a dry bottle in the ice-chest. This standard, in carefully purified toluene, was found to keep from 3 to 4 weeks without reduction of the colour produced when an equal volume of chloroform and a drop of 2% picric acid in chloroform were added. The standard was renewed every 2 weeks, comparison being made with the previous standard. The degree of colour production was very constant.

x-positive base in the blood. The volume of the blood sample was brought to 20 c.c. with 0·9% NaCl; 4 c.c. of 10% NaOH, and then 5 or 6 c.c. of toluene were added; careful shaking, by circular action, was continued for 15 min. After centrifuging, a given volume of toluene extract was treated with an equal volume of chloroform and a drop of 2% picric reagent. The colours produced by positive blood samples were too pale to allow of accurate estimation, though they were definite. Ephedrine added to blood can be detected at a concentration of 1 μg./c.c. in an 8 c.c. sample of blood.

RESULTS

Short-term experiments

Renal ischaemia and the appearance of x*-positive base in the urine.* The right renal artery was partially obstructed in each of seven normal perineotomized bitches. Before the operation the urine of each bitch was *x*-negative, but when re-examined 4 or 5 days after operation it was *x*-positive, and remained so throughout the 3 months of observation. Control examinations of the urine of four bitches undergoing other operations yielded negative results (Table 1).

TABLE 1. The excretion of *x*-positive base in the urine following the production of renal ischaemia

	Control urine examination			Post-operative urine examination		
Dog	Sp.gr.	Result	Operation	Days after operation	Sp.gr.	Excretion of *x*. Ephedrine HCl equiv. (g./min.)
286	1018	Negative	Clip on renal artery	4	1020	$5{\cdot}7\times10^{-6}$
284	1016	,,	,,	6	1028	$8{\cdot}0\times10^{-6}$
279	1018	,,	,,	5	1025	$7{\cdot}3\times10^{-6}$
278	1020	,,	,,	7	1018	$1{\cdot}0\times10^{-5}$
277	1008	,,	,,	7	1018	$1{\cdot}2\times10^{-5}$
276	1014	,,	,,	9	1008	$1{\cdot}0\times10^{-5}$
275	—	,,	,,	9	1010	$1{\cdot}2\times10^{-5}$
280	1023	Negative	Hypophysectomy	9	1008	Negative
278	1026	,,	Perineotomy	7	1014	,,
277	—	,,	,,	8	1020	,,
276	1014	,,	,,	5	1018	,,

In order to establish the time relationship between the production of renal ischaemia and the appearance of a positive colour test in the urine, without the complication of operative and anaesthetic states, a screw compression unit was implanted around the right renal artery of a normal perineotomized bitch which had been trained to table-work. This bitch, having completed an uninterrupted recovery from operation, lay at rest on a warmed table. After control periods of urine collection and recorded blood pressure, a known compression was applied to the right renal artery of the conscious dog, and was released after a given time interval. Blood and urine samples were examined for the presence of *x*-positive base; these results and details of the experiments are given in Table 2. In the first experiment, *x*-positive base appeared in the urine during the first 16·5 min. of compression of the renal artery in amounts

TABLE 2. *x*-positive base appears in the urine of dog 273 after the production of renal ischaemia. The output is independent of the minute volume of urine

		Urine samples			Excretion of *x*.	Blood samples	
		Collection					
Time	Procedure	Began	No. of min.	Min. vol. (c.c.)	Ephedrine HCl equiv. (g./min.)	Time	Result
Exp. 1.							
10.25 a.m.	200 c.c. water by stomach tube	—	—	—	—	—	
10.40 a.m.	Urine collection and B.P. record began	10.40 a.m.	71	4·2	Nil	—	—
11.51 a.m.	1·72 mm. compression, right renal artery	11.51 a.m.	16·5	0·33	3·2 × 10^{-6}	—	—
12.11 p.m.	Compression increased to 2·15 mm.	12.11 p.m.	15	0·55	3·5 × 10^{-6}	—	—
12.16 p.m.	Compression released	—	—	—	—	—	—
Exp. 2.							
11.0 a.m.	Urine collection began	11.13 a.m.	20	0·23	Nil	—	—
		11.33 a.m.	10	0·24	Nil	11.15 a.m.	Neg.
11.43 a.m.	2·15 mm. compression right renal artery, B.P. record began	11.43 a.m.	15	0·27	9·0 × 10^{-7}	—	—
12.34 p.m.	B.P. record ended, returned to kennel, drank	11.58 a.m.	36	0·37	2·45 × 10^{-6}	—	—
		12.34 p.m.	156	0·37	6·4 × 10^{-6}	12.25 p.m.	Trace
3.15 p.m.	B.P. record and urine collection resumed	3.15 p.m.	5	2·1	9·7 × 10^{-7}	3.12 p.m.	Trace
		3.20 p.m.	6	1·8	6·0 × 10^{-6}	—	—
		3.26 p.m.	8	1·34	5·8 × 10^{-6}	—	—
		3.34 p.m.	10	0·59	5·2 × 10^{-6}	—	—
3.44 p.m.	Compression released	3.44 p.m.	10	0·36	3·5 × 10^{-6}	—	—
		4.4 p.m.	15	0·34	3·0 × 10^{-6}	—	—
		4.19 p.m.	15	0·31	4·4 × 10^{-6}	4.18 p.m.	Trace
4.30 p.m.	B.P. record ended	4.34 p.m.	10	0·28	2·6 × 10^{-6}	—	—
		4.44 p.m.	15	0·37	5·0 × 10^{-6}	—	—
		4.59 p.m.	15	0·22	2·3 × 10^{-6}	—	—
5.30 p.m.	Dog put into metabolism cage	5.30 p.m.	16½ hr.	0·21	2·7 × 10^{-6}	—	—
Next day							
10.0 a.m.	Urine collection began	10.0 a.m.	180	0·44	1·5 × 10^{-6}	10.20 a.m.	Neg.
		12.0 noon	180	0·58	Trace	—	—
		2.0 p.m.	180	0·54	Nil	3.50 p.m.	Neg.

comparable to that found in the urine during the second 15 min. of compression; excretion therefore probably began early in the compression period. In order to exclude the possibility that either the low minute volumes of urine recorded or the infusion of heparin might be associated with the appearance of *x*-positive base in the urine, the procedure was repeated with the following differences: no water was given by stomach tube, no compression was applied to the renal artery, and 18·6 mg. of heparin were injected intravenously after the first 45 min. of observation. In the 2½ hr. of observation, minute volumes of urine ranging from 0·36 to 1·64 c.c. and averaging 0·53 c.c. were recorded; no *x*-positive base was excreted. Its excretion in the previous experiment cannot therefore be attributed either to low minute volumes of urine secretion, or to the infusion of heparin, and may therefore be related to the renal ischaemia.

In the second experiment, *x*-positive base, absent before compression, again appeared in the urine during compression, and continued to be excreted for many hours after the release of compression. A trace of colour developed only in the test solutions from blood samples taken during compression. In neither of these two experiments was any rise of blood pressure recorded as the result of compression of the renal artery; this was probably due to the presence of a sound contralateral kidney (Verney & Vogt, 1943).

The next experiments were designed to discover whether the basic compound responsible for the development of the colour test was excreted by the ischaemic kidney, by the normal kidney, or by both. Its excretion by the normal kidney would be expected if the substance was present in the blood stream.

x-positive base excreted mainly by the normal kidney. Dog 273. The right renal artery was constricted by means of a silver clip of an internal diameter of 2 mm., inserted around its origin. Urine obtained by catheter 2 days before operation contained no *x*-positive base; a similar specimen 2 days after operation gave a positive colour test. On the fourth day after the first operation, the ureters were extended to the surface. The animal made an uninterrupted recovery.

The dog lay on a warmed table, and over a period of 3 hr. urine was collected from each exteriorized ureter. After 1 hr., water diuresis was produced by giving two doses of 200 c.c. of water by stomach tube at an interval of 30 min. The left normal kidney was excreting at a rate varying from 0·09 to 1·25 c.c./min. and the ephedrine colorimetric equivalent excretion of *x*-positive base varied from 20 to 60×10^{-7} g./min., independently of minute volume. The function of the right kidney was found to be much reduced, the minute volume varying from 0·04 to 0·1 c.c. Urine from this kidney contained no more than a trace of *x*-positive base. The degree of right renal ischaemia was more severe than had been intended and, under these conditions, the *x*-positive base was excreted almost entirely by the normal kidney. In order to determine whether this occurred with controlled and moderate degrees of renal ischaemia, a compression unit was inserted and the ureters extended to the surface in a second animal.

Dog 274. Four days before operation, the arterial blood pressure, measured by femoral puncture, was 126 mm. Hg, and there was no *x*-positive base present in the urine. At operation, the ureters were extended to the surface, and a fluid compression unit implanted around the origin of the right renal artery.

The dog lay on a warmed table and urine was collected from both ureters; a continuous record of the femoral arterial pressure was made, and compression was applied to the right renal artery for a period of 52·5 min. Five minutes after the renal artery was compressed, the urine flow from the left side stopped, 5 min. later the flow from the right side ceased, but began again spontaneously after 2 hr. 45 min. The cessation of flow from the left side was due to an obstruction in the ureteric tube, this was released by careful suction, and a free flow of urine then began. When the flow had settled to a steady rate the urine was collected from each side. The results are summarized in Table 3. In this experiment, urine secreted from either side was negative to the colour test before compression of the renal artery. After compression, whilst the left normal kidney was obstructed, some x-positive base was excreted by the ischaemic right kidney. After relief of the left ureteric obstruction the x-positive base was excreted in larger amount, and almost entirely by the left non-ischaemic kidney. A faint positive colour test was given by a blood sample taken during the compression period. Again there was no rise of blood pressure other than the small fluctuations associated with restlessness.

TABLE 3. The excretion of x-positive base in the urine by dog 274 after compression of the right renal artery. During left ureteric obstruction some x-positive base is excreted by the ischaemic kidney; after relief of this obstruction, the base is almost entirely excreted by the normal kidney

Time	Procedure	Urine samples, right ureter			
		Began	No. of min.	Min./vol. in c.c.	Excretion of x. Ephedrine HCl equiv. (g./min.)
11.0 a.m.	Urine collection began	11.7 a.m.	32	0·22	Nil
11.38 a.m.	Right renal artery compressed by the injection of 60 cu.mm.	11.39 a.m.	19	0·27	$7{\cdot}0 \times 10^{-7}$
12.30 p.m.	Compression withdrawn	2.30 p.m.	45	0·03	$9{\cdot}0 \times 10^{-7}$
3.0 p.m.	Left ureteric obstruction removed	3.37 p.m.	38	0·02	Trace only

Time	Procedure	Urine samples, left ureter				Blood samples
		Began	No. of min.	Min./vol. in c.c.	Excretion of x. Ephedrine HCl equiv. (g./min.)	
11.0 a.m.	Urine collection began	11.12 a.m.	27	0·12	Nil	11.5 a.m. negative
11.38 a.m.	Right renal artery compressed by the injection of 60 cu.mm.	—	—	—	Obstructed	12.15 p.m. trace
12.30 p.m.	Compression withdrawn	—	—	—	Obstructed	—
3.0 p.m.	Left ureteric obstruction removed	3.37 p.m.	10	0·65	$7{\cdot}6 \times 10^{-7}$	—
		3.47 p.m.	10	0·65	$1{\cdot}8 \times 10^{-6}$	—
		3.57 p.m.	10	0·51	$1{\cdot}9 \times 10^{-6}$	—
		4.7 p.m.	10	0·45	$2{\cdot}0 \times 10^{-6}$	—

Long-term experiments

The experiments in the previous section have shown that on the production of renal ischaemia a basic chemical compound appears in the urine. In order to study the relationship of this compound to experimental hypertension, observations were made on two dogs which were rendered hypertensive by the application of silver compression clips to the renal artery. The operative histories of these two animals are summarized below.

Dog 275. 24. x. 39, denervated carotid loop. 2. viii. 40, perineotomy. 10. viii. 40 (operation 1, Fig. 1), a split silver tube was applied to the right renal artery, inner diameter 1·75 mm. The interpolar length of the left kidney was 48 mm. Uninterrupted recovery.

17. x. 40, interpolar length of the left kidney 50·5 mm., of the right kidney, 46 mm. A split silver clip, inner diameter 2·0 mm. was applied to the left renal artery. Uninterrupted recovery (operation 2, Fig. 1).

Dog 276. 1. xii. 39, denervated carotid loop. 2. viii. 40, perineotomy. 10. viii. 40 (operation 1, Fig. 2), a silver clip of inner diameter 2·0 mm. was applied to the right renal artery. At operation, the interpolar length of the right kidney was 52 mm., that of the left 52·5 mm. Recovery was uninterrupted.

17. x. 40, clip applied to the left renal artery, inner diameter 2·25 mm. Interpolar length of the left kidney, 53 mm., of the right kidney 46 mm. Uninterrupted recovery (operation 2, Fig. 2).

After a control period in which the normal resting blood pressure of these two dogs was determined, and the urine and blood were found to contain no x-positive base, the right kidney of each dog was rendered ischaemic. After the constriction of the right renal artery the blood pressure rose, and x-positive base appeared in the blood and urine in each case. Later, the blood pressure fell rather abruptly to normal, coinciding with an increase in the specific gravity of the morning urine and a fall of the moderately raised blood urea. x-positive base, although being still freely excreted in the urine, was now absent in the blood or present only as a trace. Albumen, not accounted for by a few pus cells, was found in the urine of both dogs throughout the period of hypertension. The results are summarized in the accompanying charts (Figs. 1, 2).

Thirst and polyuria developed one week after the first operation in dog 275. It coincided with a rapid increase in weight, and granular casts were present in the urine. The fluid intake was measured for 2 days (1230 and 950 c.c.), and then water was limited to 600 c.c. in 24 hr., 100 c.c. being given at 7 a.m., 9 a.m., 1 p.m., 9 p.m. and 12 midnight; thirst was not evident. During this period the blood pressure rose, the blood urea rose, and the urine remained of low specific gravity and contained both granular and hyaline casts. The excretion of x-positive base in the urine fell abruptly. The dog lost appetite, and in view of the possible development of convulsive uraemia, it was decided to carry the experiment no further. From 5 p.m. on the eleventh day after operation fluid was freely given: 2500 c.c. were drunk overnight. The blood pressure fell sharply, and the excretion of x-positive base in the urine was rapidly resumed.

Dog 276 developed extreme thirst and polyuria 9 days after the first operation. On the twelfth day after operation the intake of fluid was 3560 c.c., and 2205 c.c. of urine were excreted in 16 hr. This thirst and polyuria coincided with a rapidly falling blood pressure, and a blood urea of 41–44 mg./100 c.c.

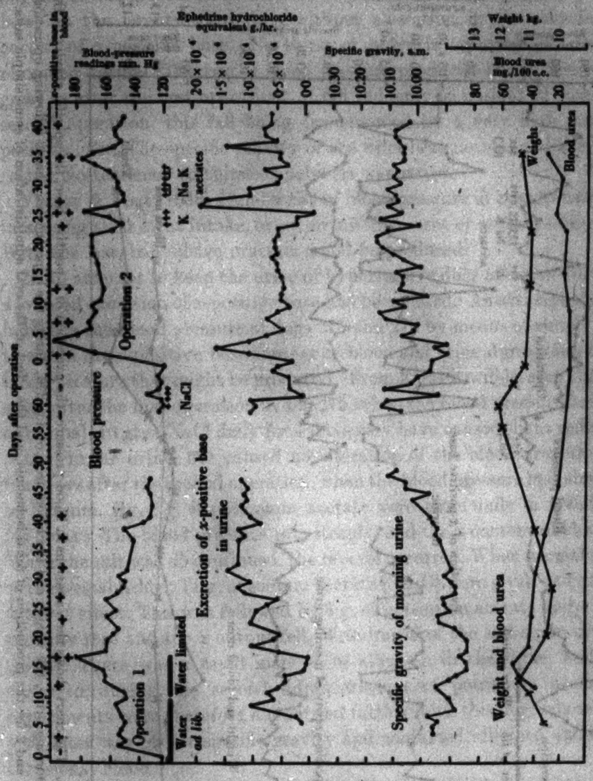

Fig. 1. The chart shows the variations in blood pressure, the excretion of *x*-positive base, the specific gravity of the morning urine, the weight and the blood urea, of dog 276 during the hypertensive phases following operations 1 and 2 for the production of renal ischaemia, and the effect of the oral administration of some simple salts.

As the blood pressure fell, the thirst and polyuria lessened. Detection of *x*-positive base in the very dilute urine was uncertain. The fact that it was being excreted during the severe polyuria was demonstrated on the twelfth day, a rather more concentrated sample of urine being obtained by means of repeated small injections of posterior pituitary extract. Fluids were restricted on the eighteenth day after operation to 800 c.c. daily, for 6 days 100 c.c. were given every 2 hr. from 8 a.m. to 10 p.m. each day. The excretion of x-positive base decreased and fell to zero on the twenty-third day, whilst the blood pressure rose to 198 mm. Hg. Although the blood urea rose from 44 to 66·5 mg./100 c.c., the specific gravity of the urine increased. Retention of x-positive base was associated with a rising blood pressure. At 12 noon on the twenty-third day water was freely given. From 12 noon to 5 p.m. 3500 c.c. of water were drunk and 1500 c.c. of urine passed, sp.gr. 1010. Water continued to be drunk.

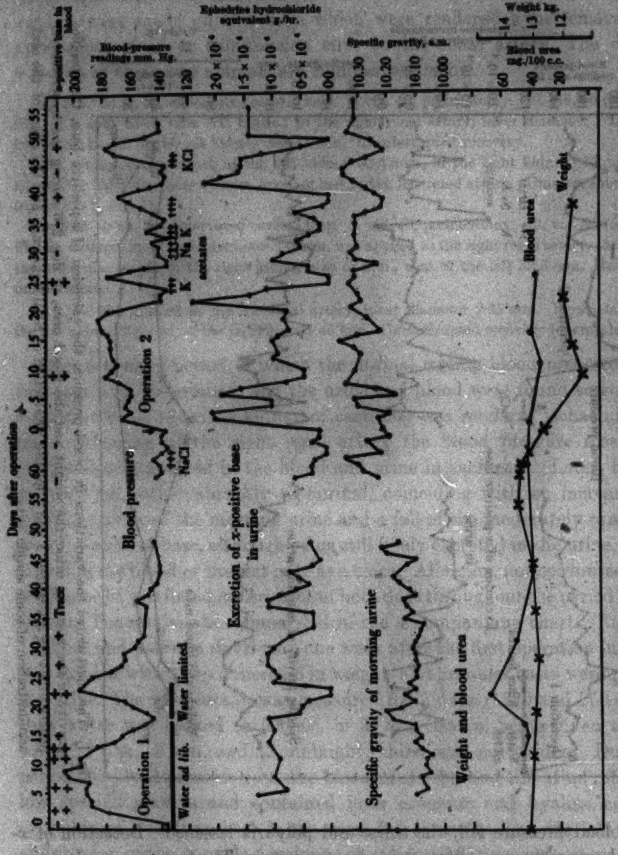

Fig. 2. The chart shows the variations in blood pressure, the excretion of x-positive base, the specific gravity of the morning urine, the weight and the blood urea, of dog 276, during the hypertensive phases following operations 1 and 2 for the production of renal ischaemia, and the effect of the oral administration of some simple salts.

Between 5 p.m. and 10 p.m. 120 c.c. of urine, sp.gr. 1010, were passed. Overnight, 650 c.c. of urine were voided, sp.gr. 1012. The blood pressure fell more gradually than in dog 275.

After the second operations hypertension again developed in both dogs. In dog 275 it was greater than after the first operation, and fell steeply to a lower level on the twenty-third day, without alteration in the morning rate of output of x-positive base. No thirst or polyuria occurred. The blood urea remained normal. The rise of blood pressure produced in dog 276 was not as great as after the first operation. It fell suddenly to normal 22 days after the second operation, this fall being accompanied by a very high output of x-positive base. The specific gravity of the urine was normal and there were no casts. No tendency to thirst or polyuria was noted.

Verney & Vogt (1938) found a rise of blood pressure in hypertensive dogs on increasing their meat intake, or by giving them urea or excess sodium chloride. With the last, convulsive uraemia could be produced.

In an attempt to keep the urine of hypertensive dogs alkaline with acetates, a lowered excretion of x-positive base had been noted. An attempt was made to influence the blood pressure of dogs 275 and 276 by means of simple salts, and to watch the x-positive base content of blood and urine during any changes of blood pressure that might be produced. From Fig. 1 it will be seen that at a late stage after the first operation in dog 275, when the blood pressure had returned to normal, 10 g. of NaCl daily for 4 days may have caused slight reduction in x output in the urine, but caused no alteration of the blood pressure. Twenty-four days after the second operation, when the blood pressure had already fallen to 150 mm. Hg, 8 g. of potassium acetate were given daily in bread and milk for 3 days. The blood pressure rose steeply, and the x content of the urine fell. When the salt was discontinued, the reverse occurred. When normal conditions were re-established, 11 g. of sodium acetate ($3H_2O$) were given daily for 3 days, without effect. This was followed by 8 g. of potassium acetate daily; the blood pressure rose and the x output fell. Withdrawal of the salt again reversed the results. There was a rapid increase of albumen in the urine, and casts reappeared during the second administration of potassium acetate; these experiments were therefore not carried further with this dog, whose urine one year later was of low specific gravity and contained albumen and casts with occasional blood.

Dog 276, at a late stage after the first operation, when the blood pressure had been normal for 20 days, showed no change in the blood pressure, but a lowered hourly output of x on giving 10 g. NaCl daily for 3 days. Twenty-one days after the second operation, the blood pressure fell precipitately to normal. After 3 days of normal blood pressure 8 g. of potassium acetate were given daily for 3 days in bread and milk. The blood pressure rose to 176 mm. Hg and the x output in the urine fell to zero. Withdrawal of the salt resulted in a rapid fall of blood pressure and a large *x* output in the urine. Sodium acetate, 11 g. daily, then produced very little effect. Repetition of the potassium acetate was now much less effective. Sodium chloride was then given (10 g. daily for 4 days), producing results comparable with the first administration of potassium acetate. Potassium chloride was then given, 13 g. for 2 days, and 6·5 g. on the third day, with similar effect.

These results suggest that there is a period of instability of blood pressure and kidney function which continues after the blood pressure has fallen to normal.

SUMMARY

The results of the short-term experiments may be summarized as follows:

(1) Partial occlusion of the right renal artery results in the appearance in the urine of a basic chemical compound which can be extracted from alkaline solution by toluene, and gives an intense yellow colour in chloroform-toluene mixture, with picric acid. This compound is found to appear in the first few minutes after the renal artery has been compressed, and to continue to be excreted for some hours after the release of compression.

(2) In two dogs in which the ureters were exteriorized, this base was excreted mainly by the normal kidney, suggesting that the substance was excreted by the ischaemic kidney into the blood stream.

(3) In two instances, blood samples taken during compression of the renal artery showed a trace of colour reaction when extracted and tested, whereas control blood samples before compression showed no development of colour when similarly treated.

The results of long-term observations on dogs 275 and 276 are summarized as follows:

(1) The production of renal ischaemia and hypertension by operative methods was accompanied by the appearance in the blood and urine of a basic compound termed *x*.

(2) The fall of the blood pressure to normal synchronized with the disappearance of this compound from the blood, but its presence in the urine continued.

(3) Raising the blood pressure by limitation of fluid intake and by the administration of simple salts was accompanied by a rise in *x* in the blood and by a fall of *x* in the urine, these results being reversed by removal of the causal agent.

(4) The potassium ion was found more effective than the sodium ion, and the chloride ion more potent than the acetate ion in the production of these changes.

(5) There is no evidence that the thirst and polyuria were caused by the chemical compound *x*, nor that *x* was responsible for the production of hypertension.

I would like to express grateful thanks to Dr E. B. Verney, who, at great expenditure of his time, performed all the surgical operations and femoral punctures described in this paper, and constantly gave his help in the collection of blood and urine samples, being responsible for the care and supervision of all dogs throughout this work.

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THE PROPERTIES AND PHARMACOLOGICAL ACTION OF A BASE LIBERATED BY ALKALINE HYDROLYSIS OF NORMAL URINE, COMPARED WITH THOSE OF A SIMILAR BASE FOUND FREE IN THE URINE OF DOGS WITH RENAL ISCHAEMIA

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The appearance of a new base, detected by a colour test, in the urine of dogs shortly after one kidney had been rendered ischaemic by surgical means, has been described (Lockett, 1946). No such base was found free in the urine of normal dogs or in human urine.

This base has been found to be volatile in steam from alkaline solution. In a late stage of renal hypertension, larger amounts of the base were detected colorimetrically in the steam distillates than in the original urine. This led to the discovery that alkaline hydrolysis of these urines (pH 12·0, NaOH, 100° C. for 5 min.) liberated an amount of base colorimetrically equivalent to the excess yields of the steam distillates, and that similar hydrolysis of normal dog or human urine yielded base giving the same colour test. To facilitate description, urine from dogs having an ischaemic kidney is termed 'clamp urine', and bases giving the positive colour test, x, are termed 'x-positive'. The paper is divided into two sections: in the first, comparison is made between the physical and chemical properties of the x-positive base free in clamp urine, and the base liberated by alkaline hydrolysis of normal urine; in the second part, the pressor activities of the bases from clamp and normal dog urine are compared, and the pharmacological actions of the latter base examined. No differences have been detected between the properties of these bases, and there is strong indication for the existence of a single complex x-positive base which cannot be isolated from solution unchanged.

METHODS

The collection of urine

Normal urine

Human. Pooled normal urine was collected from convalescent surgical patients in the wards of Addenbrooke's Hospital. Phenol or purified toluene was used as bacteriostatic.

Bitches. Metabolism cages were used. In order that a total collection for the 24 hr. should be made from each animal, those that had not urinated were catheterized, aseptically, each morning, before they were put out on the play-roof for 3 hr.

Urine from bitches with renal ischaemia

Renal ischaemia was brought about by the implantation of small silver clips around the origin of the right renal artery, according to the method described by Verney & Vogt (1938). Urine was collected from these as from normal bitches.

The preparation of aqueous concentrates of x*-positive base*

Owing to the properties of *x*-positive base, some special methods had to be used for the preparation of these concentrates.

Concentration of urine. Preliminary concentration of normal urine to not less than ½ vol., at a temperature not exceeding 90° C., no direct heat being applied, did not lower the yield of *x*-positive base by more than 4%. Urine from bitches with renal ischaemia was not concentrated.

Alkaline hydrolysis and distillation in steam. Alkaline hydrolysis and steam distillation were performed in one operation. The cooled and filtered concentrate of normal urine was brought to a pH just blue to alizarin as external indicator, with 20% NaOH, and was distilled in steam, slowly, to avoid severe frothing. The highest yields were obtained when multiple fractions of volume less than 300 c.c. were worked; when larger volumes were distilled, e.g. 1 l., the yield fell 10–15%, and the distillate was coloured faintly yellow.

Urine from bitches with renal ischaemia was brought to pH 11·5 with Na_2CO_3 and was distilled in steam without preliminary concentration.

The concentration of the distillate. As *x*-positive base is unstable to acid, use was made of the fact that such base shows an optimum pH for distillation in steam. The base was concentrated quantitatively either in a high vacuum, or in an atmosphere of nitrogen, at an internal temperature not exceeding 32° C., without the addition of acid. At higher temperatures there was a fall in the total colorimetric equivalent ephedrine content, and the solution became yellow and turbid.

By these methods a highly concentrated, clear and colourless aqueous solution of *x*-positive base was obtained and separated from simpler contaminating bases. Ammonia, methylamines, added piperidine and aliphatic amines passed quantitatively into the distillate, no trace of them being found in the final solution of *x*-positive base.

The colorimetric estimation of x*-positive base*

The method for estimation of *x*-positive base in urine has been described (Lockett, 1946), and this has been adapted to estimate *x*-positive base in all aqueous solutions, since it was found by dilution experiments to give constant results up to an ephedrine colorimetric equivalent of 0·1 g./vol. of fluid used for estimation. At higher levels the results were unsatisfactory. A high concentration of ammonia in solution was found to interfere with the colorimetric estimations; ammonia correction curves were therefore constructed.

Two standard methods of estimation have been adopted: the results obtained by both methods were identical.

In estimations on 10 *c.c. of fluid*, 2 c.c. of 10% NaOH were added, and the whole was shaken with 3 c.c. of purified toluene for 10 min. After centrifuging, to 1·5 c.c. of toluene extract 1·5 c.c. of $CHCl_3$ and 0·1 c.c. of 2% picric in $CHCl_3$ were added. The resulting yellow *x*-positive solution was compared in a colorimeter with an ephedrine standard, prepared either by extraction of an aqueous solution of the hydrochloride or by direct solution of crystalline ephedrine, the amount of x-positive base present being measured as a colorimetric equivalent ephedrine concentration in g./c.c.

In estimations on 25 c.c. of fluid, 4 c.c. of 10% NaOH were added, and the whole was shaken with 6 c.c. of toluene for 10 min., centrifuged, etc.

The determination of the optimum pH for distillation in steam

A known volume of urine or urine concentrate was brought to a given pH with NaOH or Na_2CO_3, and the x content of successive fractions of distillate was determined by one of two methods which gave comparable results: (*a*) after removal of the ammonia by concentrating to small volume, as described above, or (*b*) without concentration, the ammonia correction being applied.

To determine the optimum pH for the distillation of x-positive base which had been liberated by alkaline hydrolysis, distillate concentrates were added to urine concentrate which had been exhaustively distilled in steam until no further x-positive base passed into the distillate, cooled, neutralized, and treated with ammonium sulphate in amount equivalent to the original NH_3 content of the concentrate.

Pharmacological methods

The procedures by which cats were anaesthetized, chloralose anaesthesia induced, records of blood pressure made, injections given, decerebration and acute sympathectomy performed were identical with those already described (Lockett, 1946).

Destruction of the brain. The spine of the axis and the dorsal arch of the atlas were removed, the occipito-atlantoid membrane was incised, and after artificial respiration had been started, a thin, blunt-ended rod was passed through the foramen magnum into the cranial cavity. The wound was closed by suture.

Evisceration. The coeliac axis, superior and inferior mesenteric arteries were isolated and divided between ligatures; the oesophagus was tied just below the diaphragm, and the rectum ligatured; both were cut across and the viscera lifted to drain them of blood. The structures in the portal fissure were divided between double ligatures. The alimentary tract and spleen were then removed. The kidneys and suprarenals were carefully isolated, vessels were divided between ligatures, and the organs then removed. The abdomen was closed by suture.

Records of uterine movement and urine flow. One uterine horn was withdrawn through a small incision in the mid-line of the lower abdomen, its mesentery divided between ligatures. A ligature was tied around the uterus at the origin of the horns, without interfering with the uterine vessels. This served later to fix the adjacent origin of the uterine horn, the movements of which were to be recorded. A purse-string suture was passed through the edges of the abdominal incision and a fine thread attached to the free end of the horn. A glass cylinder, diameter 2 cm., having its lower edge everted and the upper reduced and sealed to a short piece of narrow-bore tubing, was used to keep the uterine horn moist and warm. The fine thread was passed into the cylinder to emerge through the narrow glass tubing, and the everted edge of the cylinder fixed within the abdominal incision by means of the purse-string suture. The uterine ligature was included within the suture and suitably fixed so as to immobilize the uterus below the opening into the cylinder. The cylinder was clamped in a vertical position, and the fine thread was passed over a system of pulleys and connected with a frontal writing lever by which the uterine movements were recorded on a drum.

Urine secretion was recorded by opening the fundus of the bladder, passing a self-retaining catheter through the vesical opening of the urethra, closing the incision into the bladder by suture, and registering the drops of urine.

Preparation of solutions for injection

x-positive base. This was a 100% yield concentrate (see below) prepared by the method previously described, from 60 l. of normal urine; final volume 19 c.c. This solution was treated with blood charcoal and filtered through kieselguhr to ensure that no nicotine or base A remained, though this normally passed over quantitatively into the distillate during concentration: *x*-positive base is not adsorbed on blood charcoal in the absence of free ammonia (Lockett, 1944). The volume was then brought to 80 c.c. with water, and NaCl was added to 0·9%. This solution was used for all the experiments on the site of pressor action of the base from normal human urine, which were done in the course of 3 days, except cat Exp. 4, for which a similar solution was prepared from 20 l. of urine, this being labelled (*b*).

The content of *x*-positive base in these two solutions, measured as a colorimetric equivalent concentration of ephedrine hydrochloride in g./c.c., were $1\cdot1 \times 10^{-3}$ and $1\cdot4 \times 10^{-3}$ (*b*). Controls on both solutions showed total removal of both colour-producing compound and pressor activity by benzoylation.

Adrenaline tartrate. Solutions of the pure salt (Burroughs Welcome) in 0·9% NaCl were prepared immediately before use, strength 20 μg./c.c.

Atropine sulphate. Solutions contained 1 mg./c.c.

RESULTS

A comparison of the physical and chemical properties of the x*-positive bases of clamp and normal urine*

The *x*-positive base of clamp urine and that liberated by alkaline hydrolysis from normal urine showed identical properties:

(*a*) *The two* x*-positive bases from clamp and normal urine had the same optimum pH for volatility in steam*

The pH optimum for the distillation in steam of the *x*-positive base of clamp urine was found to be 11·5 (Na_2CO_3). The maximum yields of *x*-positive base from normal urine were constantly recorded at pH 13·0 (NaOH), and this base appeared to distil more slowly in steam. Such results were compatible with the theory that the base from normal urine was being liberated by alkaline hydrolysis. This was confirmed, for when a concentrate of *x*-positive base from normal urine was added to urine concentrate freed of the precursor, and the optimum pH for the distillation in steam was redetermined, the value obtained was pH 11·5 (Na_2CO_3).

Comparing Tables 1 A and 1 B, it is evident that there is a close parallel in the rates of distillation and the yields obtained at various pH values between the two *x*-positive bases of clamp and normal urine. Since both these two *x*-positive bases show stability to heating, sealed at 100° C. for 30 min., with 10% NaOH, the pH optimum of 11·5 (Na_2CO_3) for both bases is a true value, and is indicative of bipolarity.

Use was made of the fact that at very low alkalinity no *x*-positive base passes over with the water vapour (Table 1) in the special method described for the quantitative concentration of *x*-positive base in the steam distillates, since the behaviour of these bases towards acids, precipitating reagents, and organic solvents made the usual methods unsuitable.

(*b*) *The behaviour of* x*-positive base towards acids, precipitating reagents, and organic solvents*

No discrepancy was found in the behaviour of the bases of clamp and normal urine in the large number of tests briefly summarized below.

TABLE I. A comparison of the rates of distillation in steam, and the yields obtained at varying pH, between the x-positive base of clamp urine and that from normal urine

	pH	Vol. of urine distilled c.c.	x in colorimetric equivalent ephedrine concentration, g./total vol. In vol. urine distilled	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Total recovery	Yields % This experiment	Other experiments	Average
A. Na_2CO_3	8·0	80	$8\cdot0\times10^{-4}$	$1\cdot8\times10^{-5}$	$1\cdot6\times10^{-5}$	$1\cdot3\times10^{-5}$	$1\cdot3\times10^{-5}$	$1\cdot0\times10^{-5}$	$7\cdot0\times10^{-5}$	8·6	6·0, 5·0	6·5
	9·0	50	$2\cdot3\times10^{-4}$	$2\cdot7\times10^{-5}$	$2\cdot7\times10^{-5}$	Trace	Trace	None	$5\cdot4\times10^{-5}$	23·5	15·0, 20·0	19·5
	10·0	53	$2\cdot9\times10^{-4}$	$1\cdot4\times10^{-4}$	$3\cdot0\times10^{-5}$	Trace	None	None	$1\cdot7\times10^{-4}$	59·0	50·0, 51·0	53·0
	11·0	100	$2\cdot9\times10^{-4}$	$1\cdot6\times10^{-4}$	$2\cdot8\times10^{-5}$	Trace	None	None	$1\cdot9\times10^{-4}$	67·0	66·0, 55·0, 56·0	61·0
	11·5	140	$7\cdot0\times10^{-4}$	$5\cdot7\times10^{-4}$	$7\cdot3\times10^{-5}$	$3\cdot1\times10^{-5}$	Trace	None	$6\cdot7\times10^{-4}$	96·0	96·0, 97·0	96·0
	12·0	140	$4\cdot0\times10^{-4}$	$3\cdot9\times10^{-4}$	None	None	None	None	$3\cdot9\times10^{-4}$	96·0	90·0, 92·0	93·0
NaOH	13·0	65	$2\cdot0\times10^{-4}$	$8\cdot6\times10^{-5}$	None	None	None	None	$8\cdot6\times10^{-5}$	44·0	50·0	47·0
B. Na_2CO_3	9·0	100	$9\cdot0\times10^{-4}$	$1\cdot4\times10^{-4}$	$6\cdot0\times10^{-5}$	$4\cdot0\times10^{-5}$	Trace	None	$2\cdot4\times10^{-4}$	27·0	24·0	25·5
	10·0	100	$9\cdot0\times10^{-4}$	$2\cdot2\times10^{-4}$	$9\cdot4\times10^{-5}$	$6\cdot4\times10^{-5}$	$2\cdot1\times10^{-5}$	None	$4\cdot0\times10^{-4}$	44·0	51·0	47·5
	11·0	100	$9\cdot0\times10^{-4}$	$5\cdot0\times10^{-4}$	$7\cdot0\times10^{-5}$	$6\cdot4\times10^{-5}$	Trace	None	$6\cdot1\times10^{-4}$	68·0	69·0	68·5
	11·5	100	$9\cdot0\times10^{-4}$	$7\cdot3\times10^{-4}$	$1\cdot0\times10^{-4}$	$5\cdot0\times10^{-5}$	None	None	$8\cdot7\times10^{-4}$	97·0	96·0	96·5
	12·0	100	$9\cdot0\times10^{-4}$	$8\cdot0\times10^{-4}$	Trace	None	None	None	$8\cdot0\times10^{-4}$	89·0	88·0	88·5
NaOH	13·0	100	$9\cdot0\times10^{-4}$	$3\cdot8\times10^{-4}$	None	None	None	None	$3\cdot8\times10^{-4}$	42·0	53·0	47·5

On adding acid and evaporating, either on a water-bath or at reduced temperature and pressure, in the presence or absence of atmospheric oxygen, only a very small fraction of the total original colorimetric equivalent of ephedrine present in the distillate was found in the residues. Since these bases were not volatile from neutral or acid solution, instability towards acids was indicated. The acids used were: hydrochloric, sulphuric, acetic and oxalic.

Attempts to precipitate the bases by picric, tannic, phosphomolybdic and phosphotungstic acids, lead acetate, mercuric chloride and iodide, silver and copper salts, baryta and potassium bismuth iodide were unsuccessful. Either the bases were unaffected, or they disappeared but could not be recovered by decomposition of the precipitates.

These bases could be extracted from alkaline solution by benzene, toluene and chloroform, much less readily by ether, and not at all by petrol ethers. When the solution of the base in the organic solvent was shaken with any dilute aqueous acid, the organic solvent no longer gave the colour test, i.e. the base had been removed; if the aqueous acid solution was now made alkaline and re-extracted, only a very small amount of the original colour-producing compound appeared in the extract. This had been shown not to be due to the extraction of only a small part of the base present; alteration of the base had occurred.

The bases could not be dried or concentrated in organic solvents. They were volatile in the vapours of ether, alcohols, chloroform, benzene, and toluene, even in the presence of non-volatile acids. Their ability to produce the x colour with picric acid decreased in organic solvents in the course of a few hours, especially on drying, and despite extreme care in the purification of the solvents. The drying agents tried were: anhydrous salts, Na_2SO_4, $NaHCO_3$, K_2CO_3, $CaCO_3$, $CuSO_4$, $MgSO_4$, and also NaOH, KOH, CaO.

Numerous attempts to precipitate salts of the base in organic solvents failed. The acetyl and benzoyl derivatives were unstable, and the bases were altered by evaporation to dryness.

(c) *The changes occurring in* x*-positive base on evaporation to dryness*

The water-clear concentrates of x-positive base from clamp or normal urine had pressor action. Evaporation to dryness, under the conditions previously described for the concentration of the distillate, left approximately 1 mg. of white deliquescent solid per litre of original urine. Re-solution of this residue in $CHCl_3$ and other non-polar solvents, after 15–30 min. drying, gave 100% recovery of the total x colour originally present in the distillate; re-solution in water gave a 17% recovery. After 1 or 3 hr. drying, re-solution in $CHCl_3$ gave a 50% recovery, re-solution in water less than 5%.

Before x-positive base had been taken to dryness there was no separation of platinichlorides or other salts even at very high concentration of the base, and no primary amino-nitrogen could be detected. When the base had been dried and redissolved in water, primary amino-nitrogen was present and salts separated at moderate concentration. Drying had therefore altered the base.

(d) *The yields of* x*-positive base obtained in distillate concentrates from clamp and normal urine*

A further similarity between the x-positive bases of clamp and normal urine was found in the yields obtained in the final distillate concentrates from either source. These are given in Table 2, the base being measured as a colorimetric equivalent ephedrine concentration in g./l. of urine.

These concentrates, leaving a residue of approximately 1 mg. of solid/l. of original urine, had a colorimetric equivalent ephedrine yield of $1\cdot0$–$1\cdot4\times10^{-3}$ g./l. of original urine. They could not therefore be expected to contain a high proportion of impurities. The properties of x-positive base made the isolation of crystalline derivatives of the unchanged base impossible, and the base itself was altered by evaporation to dryness. For these reasons a series of column adsorption experiments were undertaken for the enumeration of the x-positive bases in the final distillate concentrates, and to determine whether pressor activity and x colour production were properties of the same compounds; x-positive base from normal and clamp urine again showed identical properties.

Table 2. Yields of x-positive base from clamp and normal urines

Urine			Yield of x-positive base in g./l. colorimetric equivalent ephedrine concentration	
Source		Sp.gr.	Higher range	Lower range
Dog:	Normal	1024–1032	$1\cdot2$–$1\cdot5\times10^{-3}$	$0\cdot66$–$0\cdot81\times10^{-3}$
	Clamp	1020–1036	$1\cdot3$–$1\cdot7\times10^{-3}$	$0\cdot64$–$0\cdot87\times10^{-3}$
Human:	Normal	1014–1027	$1\cdot0$–$1\cdot4\times10^{-3}$	$0\cdot53$–$0\cdot75\times10^{-3}$

In each case the yields fell into a higher and a lower range. Quantitative concentration of the distillates in respect of their total x content showed a 100 or a 50% recovery in the final concentrate. The lower range, 50% yield concentrates, showed doubling of their x content (expressed as a colorimetric equivalent ephedrine concentration) on standing with alkali at room temperature; this was never shown by final concentrates of the higher range, 100% yield, but the x content of these can be accurately halved in the course of 5–6 hr. by standing with one drop of dilute mineral acid at room temperature; the process is then reversed with alkali. This phenomenon has also been demonstrated with the x-positive base of clamp urine before distillation.

(e) *Evidence indicating the existence of a single* x-*positive base in the final distillate concentrates*

In a long series of column adsorption experiments only one x-positive zone could be found, and throughout this zone pressor effect and colour production remained parallel (Lockett, 1944), and eluates from all parts of the zone had peripheral action. If more than one x-positive base were present, then these bases were either simultaneously adsorbed in a constant fixed proportion on varying adsorbents, at varying pH, or they were adsorbed in zones, possibly overlapping; but even then the bases must all have a pressor action exactly proportional to their colour production.

Benzoylation removed all compounds responsible for both colour production and pressor activity from the distillate concentrates and from eluates of all sections of the adsorption zone; hydrolysis of the precipitated material produced no compound having either pressor activity or colour production.

Extraction of x-positive base paralleled the extraction of the pressor compound, both in the distillate concentrates and in the eluates. No separation could be effected by the use of different solvents. The disappearance of x colour reaction was paralleled by the loss of pressor activity, both when the base was passed from aqueous alkaline solution to an organic solvent and then back to dilute acid, and also on the addition of acid to an aqueous solution of the base.

The existence of an optimum pH for distillation in steam can be explained as an optimum for a given mixture of bases, but conditions resulting in the quantitative concentration of all x-positive base ensured that all components of such a mixture were present in the final concentrate. All attempts to dissociate x colour from pressor activity, or to demonstrate pharmacologically or chemically more than one such base in these distillate concentrates failed.

Conditions resulting in the reversible doubling of the colorimetric equivalent ephedrine concentration of the 50% yield concentrates and halving of that of the 100% yield concentrates have been described. The theory that this may be due to simple bimolecular polymerization is supported by the fact that it occurred without alteration in pressor activity.

Such evidence as is summarized above strongly indicates the existence of a single complex x-positive base in the final distillate concentrates. Owing to the properties of this base, the pharmacological action was necessarily studied in solution.

The pressor activity of x*-positive base*

Colorimetrically equivalent amounts of the x*-positive base from clamp and normal dog urine have equal pressor activity*

A distillate concentrate derived from the total urine (4600 c.c.) for 5 days from six normal dogs had a volume of 32 c.c. and a colorimetric equivalent ephedrine hydrochloride concentration of $6{\cdot}4 \times 10^{-4}$ g./c.c. A similar distillate concentrate from the total urine (12,090 c.c.) of seven dogs which had unilateral renal ischaemia had a final volume of 73 c.c., and a colorimetric equivalent ephedrine hydrochloride concentration of $4{\cdot}0 \times 10^{-4}$ g./c.c. NaCl was added to each to 0·9%, and both concentrates were treated with blood charcoal, to remove any possible trace of base A (Lockett, 1944), and were filtered through kieselguhr.

The pressor activities of these solutions were compared on a cat weighing 2·5 kg. The results are illustrated in Fig. 1. 5·0 c.c. of the distillate concentrate from normal dog urine equated with 8·0 c.c. of the distillate concentrate from ischaemic dog urine; each of these contained a colorimetric equivalent of ephedrine hydrochloride of $3{\cdot}2 \times 10^{-3}$ g. in the volume injected.

Colorimetrically equivalent amounts of the x-positive bases from these two sources have therefore similar pressor activity. The next experiments described were undertaken in order to gain some indication of the site of pressor action of the x-positive base, and to compare its activity with that of adrenaline.

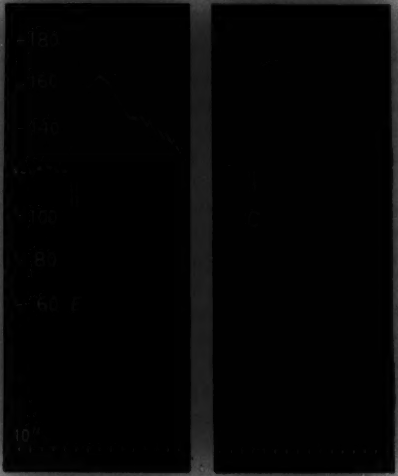

Fig. 1. The tracings show the responses of the arterial blood pressure to the injection of the following distillate concentrates: *C*, 5 c.c. of that derived from normal dog urine by alkaline hydrolysis; *E*, 8 c.c. of that from the urine of dogs which had renal ischaemia. The two solutions contained colorimetrically equivalent amounts of x-positive base in the volume injected.

Some pharmacological actions of x-*positive base from normal human urine*

Cat 1 (weight 2·4 kg., simultaneous records of blood pressure, uterine movements and urine flow). The responses under chloralose anaesthesia to 10 μg. of adrenaline tartrate and 1·5 c.c. of the x-positive solution are shown in Fig. 2*a* and *b*, and Table 3. The x-positive solution produced a rather larger rise in blood pressure than 10 μg. of adrenaline tartrate, but whereas the latter inhibited uterine movements, the x-positive base did not; this was confirmed, for 3 c.c. of the x-positive base solution produced a supramaximal rise in blood pressure, yet caused no change in uterine movements. There was no appreciable effect on urine flow; it was increased with both solutions during the period of raised blood pressure only.

After 2·5 mg. of atropine sulphate had been given intravenously, the response to 1·5 c.c. of the unknown was considerably less than that to 10 μg. of adrenaline (Fig. 2*c*, *d*) (see also cats 2 and 3, Table 3).

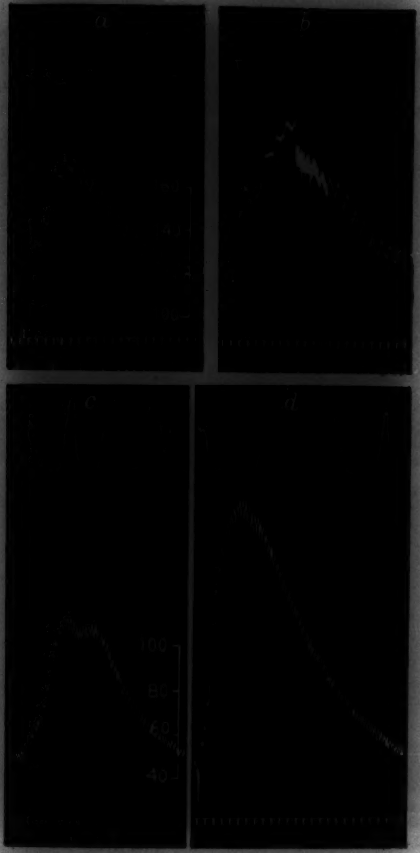

Fig. 2. The figure shows the effect of the following intravenous injections on the tracings of uterine movements, blood pressure, and urine flow: (*a*) and (*d*) 10 μg. adrenaline tartrate; (*b*) and (*c*) 1·5 c.c. of *x*-positive solution. Between (*b*) and (*c*), 2·5 mg. of atropine sulphate were given intravenously. Time marker, 5 sec.

Cat 2 (weight 2·7 kg.). The blood-pressure response to 10 μg. of adrenaline tartrate was a little smaller than that to 3 c.c. of *x*-positive base. After 3 mg.

of atropine sulphate, the responses to both were increased; 10 μg. of adrenaline tartrate was now at least as active as 3 c.c. of *x*-positive base (Table 3).

Cat 3 (weight 2·9 kg.). The responses under chloralose anaesthesia to 20 μg. of adrenaline tartrate and 2·5 c.c. of *x*-positive base were not greatly different (Table 3 and Fig. 3*a*, *b*). After total acute sympathectomy *x*-positive base was still active (Fig. 3*c*, *d*). After evisceration including the removal of both kidneys and suprarenals, while there was still a great response to 10 μg. of adrenaline (Fig. 3*e*), that to 4 c.c. of *x*-positive base was almost negligible (Fig. 3*g*). The injection of 2 mg. of atropine sulphate now had no appreciable effect on the results (Table 3).

TABLE 3. A comparison of the pressor action of *x*-positive base from normal human urine with that of adrenaline

Cat	Solution injected	Blood pressure, mm. Hg.		Conditions	Fig.
		Base-line	Rise		
1	10 μg. adrenaline	115	56	Chloralose anaesthesia	2*a*
	1·5 c.c. *x*-positive base	115	74		2*b*
	1·5 c.c. *x*-positive base	41	63	After 2·5 mg. atropine	2*c*
	10 μg. adrenaline	38	119		2*d*
2	10 μg. adrenaline	112	56	Chloralose anaesthesia	
	3 c.c. *x*-positive base	112	66		
	3 c.c. *x*-positive base	63	98	After 3 mg. atropine	
	10 μg. adrenaline	73	99		
3	20 μg. adrenaline	143	31	Chloralose anaesthesia	3*a*
	2·5 c.c. *x*-positive base	133	40		3*b*
	10 μg. adrenaline	42	84	After total acute sympathectomy	3*c*
	2·5 c.c. *x*-positive base	46	112		3*d*
	1·25 c.c. *x*-positive base	48	55		
	10 μg. adrenaline	46	112	After evisceration including kidneys and adrenals	3*e*
	4 c.c. 0·9 % NaCl	47	31		3*f*
	4 c.c. *x*-positive base	54	46		3*g*
	4 c.c. *x*-positive base	62	54	After 2 mg. atropine	
	10 μg. adrenaline	66	106		
4	2 c.c. *x*-positive base (*b*)	150	59	Chloralose anaesthesia left splanchnic cut, right suprarenal out	
	2 c.c. *x*-positive base (*b*)	137	54		
5	10 μg. adrenaline	124	72	Chloralose anaesthesia	
	2 c.c. *x*-positive base	123	57		
	10 μg. adrenaline	78	128	After 3 mg. atropine and decerebration	
	2 c.c. *x*-positive base	82	94		
	10 μg. adrenaline	86	56	After brain destroyed	4*a*
	2 c.c. *x*-positive base	84	82		4*b*
	10 μg. adrenaline	60	15	After 30 mg. ergotoxine	4*c*
	2 c.c. *x*-positive base	62	29		4*d*

This experiment showed that the pressor action of *x*-positive base is effective mainly in the splanchnic area, or on the kidneys.

Cat 4 (weight 2·1 kg.). The response to 2 c.c. of *x*-positive base was determined under chloralose anaesthesia (Table 3). The left splanchnic nerve was

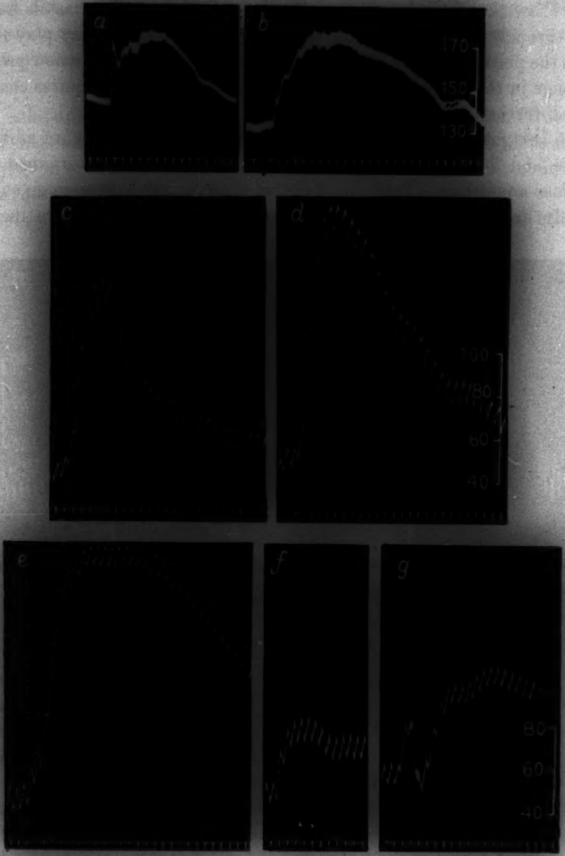

Fig. 3. The tracings show the changes in arterial blood pressure after the following injections: (*a*) 20 μg. adrenaline tartrate; (*b*) 2·5 c.c. of *x*-positive base solution; (*c*) 10 μg. of adrenaline tartrate; (*d*) 2·5 c.c. of *x*-positive base solution; (*e*) 10 μg. adrenaline tartrate; (*f*) 4 c.c. of 0·9 % NaCl; (*g*) 4 c.c. of *x*-positive base. Between (*b*) and (*c*), total acute sympathectomy, and between (*d*) and (*e*), evisceration which included both kidneys and suprarenals, were performed. Time marker, 5 sec.

cut, and the right suprarenal removed. The response was unaltered. These results show therefore that the pressor activity of x-positive base is not produced through the adrenals; they might suggest that the kidney played some part in the pressor activity, since section of one splanchnic nerve now produced no change in the response, both kidneys being intact. This requires confirmation and further study.

Cat 5 (weight 2·9 kg.). The blood-pressure responses to 10 μg. of adrenaline tartrate and 2 c.c. of the x-positive base solution were determined under chloralose anaesthesia (Table 3). 3 mg. of atropine sulphate were given intravenously and decerebration was performed; the responses were increased proportionately. The brain was then destroyed; the response to adrenaline was then found to be reduced, but that to the x-positive base was unaltered (Fig. 4*a*, *b*, and Table 3). 30 mg. of ergotoxine were then given intravenously in the course of 20 min.; the responses both to adrenaline and to x-positive base were greatly and proportionately reduced (Fig. 4*c*, *d*).

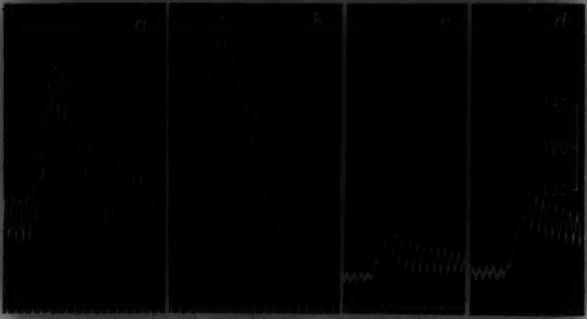

Fig. 4. The tracings show the responses of the arterial blood pressure to the injection of the following: (*a*) and (*c*) 10 μg. of adrenaline tartrate; (*b*) and (*d*) 2 c.c. of x-positive base solution. Between (*b*) and (*c*), 30 mg. of ergotoxine were given intravenously. Time marker, 5 sec.

All these results are summarized in Table 3. The pressor action of x-positive base is independent of the brain (cat 5), the adrenals (cat 4), and is unaffected by the acute removal of the sympathetic chains from the stellate ganglion to the pelvis. The pressor action is therefore peripheral. After paralysis of the motor nerve endings of the sympathetic nervous system with ergotoxine, the pressor effect of both adrenaline and x-positive base is almost abolished; it is therefore probable that the x-positive base acts on the motor nerve endings of the sympathetic nervous system in the vascular bed. The action of x-positive base differs from that of adrenaline; the blood-pressure response to x-positive base is, like that of adrenaline, increased after atropine, but to a less extent (cats 1, 2, 3), and the base does not inhibit uterine movements in the non-pregnant cat, as does adrenaline. These last observations suggest that *x*-positive base may be without action on the inhibitory fibres of the sympathetic nervous system.

SUMMARY

1. A comparison has been made of the properties of a base found free in the urine of dogs with renal ischaemia, and a similar base liberated by the alkaline hydrolysis of normal urine. These bases are found to be identical in many respects, and to differ in none.

2. Methods of preparing highly concentrated solutions of these bases are given.

3. Strong indication is presented for the existence in these concentrates of a single complex base, which, however, cannot be isolated from solution unchanged.

4. The pressor activity of the base derived from the alkaline hydrolysis of normal urine was therefore studied in solution. The evidence indicates that the base acts peripherally in the vascular bed, on the motor nerve endings of the sympathetic system.

5. Colorimetrically equivalent amounts of the base found free in the urine of dogs with renal ischaemia and the base liberated by alkaline hydrolysis from normal dog urine have equal pressor activity.

I would like to express my gratitude to Dr E. B. Verney, who performed the surgical operations on the seven dogs which had renal ischaemia, and took charge of all dogs during this work. He also carried out all the surgical procedures described in the section dealing with the pharmacological activity of the base derived from normal human urine. Pure adrenaline tartrate was very kindly supplied by Burroughs Welcome Ltd.

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CONDUCTION VELOCITY AND MYELIN THICKNESS IN REGENERATING NERVE FIBRES

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All attempts to analyse the compound action potential of nerve in terms of the nerve fibres present depend upon the basic assumption that the largest fibre present conducts with the greatest velocity. While there is no reason to doubt the validity of this assumption, there is as yet no evidence to indicate that fibre diameter, and not some variable correlated with it, is the primary factor determining the conduction velocity of nerve fibres. For example, internodal distance varies linearly with fibre diameter in the nerves of adult animals (Boycott, 1904; Kubo & Yuge, 1938; Vizoso & Young, 1946), and therefore, as Hursh (1939) suggests, can be substituted for the fibre diameter in arguments about the control of velocity. Moreover, the myelin sheath thickness varies with the axon diameter (Sanders, 1946), and thus the total fibre diameter, which is equal to the axon diameter plus twice the sheath thickness, is also related to the axon diameter. Hence the assertion that any of these variables—axon diameter, total fibre diameter, myelin sheath thickness, or internodal distance—controls conduction velocity is consistent with the available experimental evidence favouring a relationship between conduction velocity and fibre diameter. No attempt has been made so far to separate these variables by comparing the conduction velocities of, say, fibres with similar axon diameters but different sheath thicknesses or internodal distances.

During nerve regeneration fibres are found which provide an opportunity of comparing, in the same animal, the conduction rates of normal medullated nerve fibres with those of similar fibres in which the normal relations between sheath thickness, axon diameter and internodal distance have been experimentally altered. The new fibres which invade the distal stump are at first thin and non-medullated, only later acquiring myelin sheaths and increasing in diameter (Young, 1942; Gutmann & Sanders, 1943). As these fibres medullate and increase in diameter during the first 100 days of regeneration, those of the proximal stump are progressively reduced; although at the end of regeneration both proximal and distal fibres regain their normal diameters (Greenman, 1913; Gutmann & Sanders, 1943). Sanders (1946) has shown that the myelin sheaths of these shrunken proximal fibres become thickened, the axons being reduced in diameter to a relatively greater extent than the whole fibres. During regeneration, therefore, there is a transitory phase in which the proximal stump contains abnormally small fibres with abnormally thick sheaths. Moreover, Vizoso & Young (1946) have shown that at the end of regeneration all the fibres of the distal stump have internodes of similar length although they are normal as regards size and myelin thickness. In the case of large regenerated fibres the internodal length is therefore considerably shorter than that of fibres of similar diameter in normal nerve.

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The experiments described in the present paper were made primarily in order to discover whether abnormal fibres of the above two types conduct at different velocities from the normal fibres from which they are derived and, consequently, what is the effect of a change in myelin thickness or internodal distance upon conduction velocity. The measurements were made upon nerves which had been allowed to regenerate for different times after interruption by crushing with fine, smooth-tipped forceps. This operation interrupts all the fibres but leaves the endoneurium largely intact, with the result that the regenerating fibres undergo little criss-crossing in the scar, and travel mostly down the pathways which they occupied before the injury (Young, 1945). Not only do more fibres succeed in crossing the scar than after nerve suture, but a more complete reconstitution of the nerve trunk occurs after crushing than after other types of injury and nerve repair (Gutmann & Sanders, 1943). The situation is therefore an ideal one for studying nerve regeneration in the absence of complicating factors—such as the shunting of fibres which invariably follows nerve suture.

During these experiments information was also obtained concerning the progressive increase in conduction velocity in the fibres of the distal stump during regeneration, which Berry, Grundfest & Campbell (1944) described as occurring after nerve suture.

METHODS

Operative technique

The animals used were adult rabbits of both sexes. No attempt was made to select animals on a basis of age, race or body weight. Under anaesthesia induced by nembutal (1 gr./kg.) and maintained by ether, and with full aseptic precautions, the peroneal nerve was exposed in the thigh and crushed firmly with smooth-tipped watchmakers forceps. A single firm crush was used, the nerve being held compressed for about 10 sec., this being sufficient to interrupt all the fibres in the nerve (see Gutmann, Guttmann, Medawar & Young, 1942). Two groups of animals were used: (*a*) in those animals from which the data of proximal stump conduction velocity were obtained the nerve was crushed about 1 cm. above the fibula; (*b*) in those which gave data for distal stump velocity the nerve was crushed at the proximal end of the thigh; in a few animals the nerve was crushed in mid-thigh. The majority of animals were treated on one side only, the normal nerve of the opposite side being used as a control where possible, but in a few cases the peroneal nerve was crushed on both sides. After operation the animals were allowed to survive for various periods before the nerves were excised for conduction velocity estimations. During the period of survival each rabbit was tested twice weekly for the return of reflex spreading of the toes, a reaction which is a reliable estimate of a return of function in muscles supplied by the peroneal nerve (Gutmann, 1942). By comparison of the amplitude of spreading on the operated and normal sides it was possible to make an estimate of the degree of functional recovery.

Estimation of conduction rate

All experiments, except a very few noted elsewhere in the text, were made upon excised nerve. The nerves were removed from the animals under nembutal anaesthesia, and soaked in Ringer-Locke solution at room temperature for 5–10 min. This was found to be effective in preventing the repetitive discharge which often occurs with freshly excised nerves (Adrian, 1930). The site of crushing could generally be identified as a slight swelling in the course of the nerve. As great a length of nerve as possible was excised both above and below the site of crushing. 8–10 cm. of nerve was generally obtained. After excision and soaking, clamps were fixed to the ends of the nerve, and the latter transferred to a warm chamber. The two clamps fitted into slots in the chamber and could be separated by means of a screw. With the nerve in position, the clamps were moved apart until the sag of the nerve under its own weight did not exceed 2–3 mm. With the nerve held in this way, the electrodes were brought into position. These were silver wires, the ends of which could be moved along the nerve by means of rods projecting through the walls of the chamber. The chamber was filled with a moist mixture of 95% O_2 and 5% CO_2 and kept at $37{\cdot}5 \pm 1^\circ$ C. (total range of variation) by means of hot water circulating within the hollow walls.

Stimuli were provided locked to the sweep of the oscillograph time-base by a trigger circuit (Schmitt, 1938). This was coupled by condensers of short time constant (50–100 μ sec.) to a power valve normally biased to cut off. The output of this valve was connected to the nerve through an internally shielded air-cored transformer (Pumphrey & Rawdon-Smith, 1937). A differential input amplifier of conventional design was used with an earth lead on the nerve near the first recording electrode. In this way the stimulus escape was kept within bounds. The amplifier output was recorded by means of a cathode-ray tube and camera (reduction 3 : 1) in the usual way.

Single sweeps, or more frequently, stationary patterns at 10 per sec. were photographed. This produced no appreciable loss of definition. Time-marking was carried out by a 500-cycle oscillator checked against time-controlled a.c. mains. By keeping the earth and recording electrodes in a fixed position, and moving the stimulating electrodes along the nerve, photographs were obtained of the action potentials at conduction distances decreasing by successive 5–10 mm. steps. In some cases the nerves were stimulated at the peripheral, in some at the central end.

On the photographs of the action potentials the distances from the shock-escape to (*a*) the first inflexion, and (*b*) the first spike, were measured by means of a travelling microscope. The latter was also used to measure the time records, and from these data a time of conduction over a certain distance could be assigned to each photograph. A graph was then plotted of time against distance of conduction, and the slope of the straight line giving the best fit to the points found by the methods of linear regression (Fisher, 1944), the regression of time on distance being calculated. The velocity could then be found as the reciprocal of the regression coefficient. In this way lines were obtained for both inflexion and spike velocities.

Histology

At the end of each experiment the nerve was removed from the chamber and cut into 1 cm. lengths. The latter were extended on pieces of library card and fixed in Flemming's osmo-chrome-acetic mixture, dehydrated, infiltrated with paraffin, sectioned at 4–5 μ and stained by the modified Weigert method described by Gutmann & Sanders (1943). The largest fibre in each nerve was then located by means of rough measurement with an ocular micrometer, and photographs were taken of the region of nerve containing it by direct projection on bromide paper at a magnification of × 1200. The axon and total diameters of the fibres were measured by means of the travelling microscope in the way described by Sanders (1946).

Sources of error

Excluding the intrinsic variation between animals, the accuracy of any correlation between fibre dimensions and conduction velocity made by the methods described is affected by errors which may be classified as follows:

Errors of preparation. The majority of measurements were made upon excised nerve. The magnitude of the effects of this treatment cannot be assessed, and it is possible that the rates recorded are lower than those occurring naturally within the body. However, as all the nerves were treated comparably, all are affected equally by this source of error. A second source of error is that of variation in the temperature of the warm chamber. Q_{10}, for mammalian fibres, is about 1·7 (Gasser, 1931), and therefore, since the temperature varied by as much as 1° C., an estimate of velocity based on a single reading might deviate by as much as 6·0%. However, since the conduction rate assigned to each nerve was a statistical estimate based on at least seven readings, and the temperature of the chamber during an experiment, although variable, did not increase or decrease regularly, this source of error is unlikely to be serious, and certainly not systematic.

Errors in the estimation of conduction rate. At the beginning and end of each experiment, the distance from the cathode to the proximal recording electrode was measured with callipers to the nearest 0·5 mm. The intervening steps by which the electrodes were moved along the nerve were measured on the rods which projected from the warm chamber. Errors of reading are therefore possible. The regression lines of time on distance of conduction, however, in all cases passed within 1 or 2 mm. of the origin. For a maximal conduction distance of 10 cm., this represents an error of 1–2%. The estimates of conduction time are most susceptible to error. The distances actually measured on each photograph were those between the deflexion of the oscillogram produced by the 'escape' of the stimulating current, and the inflexion and first spike of the action potential. The travelling microscope used in measuring the records was the instrument used by Sanders (1946) who found that its use involved a non-systematic error of ±0·07 mm. (S.D. of 100 observations). The shortest distance measured being of the order of 2 mm., this corresponds to an error of 3·5% at the shortest distances of conduction. This small error of measurement was principally the result of difficulty in judging the exact point of inflexion of the record in the magnified image seen under the microscope. An additional error in the estimation of time lay in the fact that only a 500-cycle oscillator was used to calibrate the time base. This was, however, linear over the range used. The oscillator was checked against time-controlled a.c. mains after every experiment.

Errors in the estimation of fibre dimensions. The sources of error involved in measurements of this sort have been discussed by Sanders (1946). The same precautions as those recommended in this author's paper were adopted. Since all the measurements were made on fixed and stained fibres the absolute values recorded may be somewhat less than those for the corresponding fresh fibres, but there is probably no differential change in the axon and total dimensions (Hursh, 1939). Moreover, all the nerves were treated comparably during preparation, so that all would be affected by the errors of microscopical preparation and measurement to relatively the same extent.

Total effect of errors

From a consideration of all these sources of error it is clear there are only two serious errors which can affect the results, namely, the unknown effect of removing the nerves from the body, and the alteration in fibre dimensions produced by fixation, embedding and staining. As all nerves were treated in the same way, neither of these affects comparisons between the different nerves, on which the conclusions reached are based. As regards the non-systematic sources of error listed above, an estimate of those which affect conduction rate was made in every case by calculating the standard error of *b*—the regression coefficient of time on distance of conduction. In no case did the error exceed 5% of the value of *b*.

RESULTS

The conduction rate of normal and regenerating nerve fibres

Normal peroneal fibres. Photographs of the action potentials at different distances of conduction were made in the case of each of twelve normal nerves. A sample series of photographs, obtained from a single nerve, is given in Fig. 1. The action potential of the rabbit's peroneal nerve did not show the separation into α, β, γ, δ, and *C* elevations which is seen in the saphenous nerve (Gasser & Grundfest, 1939). There were instead two main elevations corresponding to the αβ and γδ groups. In addition there was sometimes a small upward inflexion on the rising phase of the main spike, and a small *C* wave.

The conduction times and distances for the first inflexion of the action potential in these twelve nerves are shown plotted on the same graph in Fig. 2. The conduction velocities obtained for individual nerves are given in Table 1. From these data, the normal peroneal nerve has a mean inflexion velocity of 68·6 ± 1·5 m./sec. (S.E. of mean of twelve observations); similarly, the peak of the αβ wave has a velocity of 56·7 ± 1·4 m./sec. (12). Therefore, according to the present data, the largest fibre in the peroneal nerve of the rabbit conducts impulses with a velocity of about 69 m./sec. This is somewhat slower than the velocity in the cat's peroneal (108–111 m./sec.; Hursh, 1939), but is within the range found for mammalian alpha fibres (Grundfest, 1940). The interpretation of possible differences in maximal conduction velocity between different mammalian species is further discussed below.

Conduction rates of regenerating fibres proximal to the point of crushing. Inflexion and spike velocities proximal to the site of crushing were found for twenty regenerating nerves; in twelve of these the data were sufficiently extensive to be treated statistically. The action potentials obtained from regenerating proximal stumps contained similar elevations to those already described for normal nerve. In all except the late regenerates (456 and

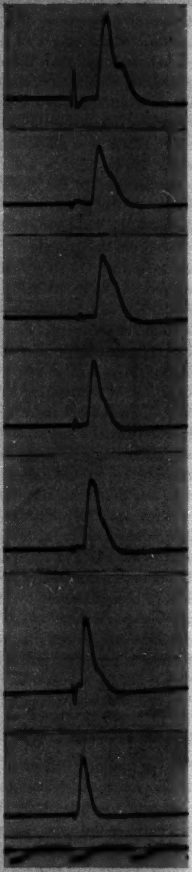

Fig. 1. Action potentials obtained from a normal nerve. Conduction distance 68 mm., decreasing by 10 mm. steps in successive photographs. Time marks = 1/500 sec.

486 days), however, there was a sharp change in velocity at the point of crushing (see Fig. 8). In the latter cases the nerves, within the limits of the experimental error, conducted at the same velocity throughout their length (see Fig. 7) and need not be considered further at present. In Fig. 3 the conduction times and distances for the inflexion of the action potential in the case of the eight proximal stumps between 56 and 123 days are shown plotted on the same graph. The conduction velocities of the individual nerves are given in Table 1. The inflexion has a mean velocity of 76·0 ± 1·8 m./sec. (S.E. of mean of eight observations); the spike, on the other hand, has a mean velocity of 66·2 ± 1·8 m./sec. (8).

Table 1. Velocities of the inflexion and spike of the action potential in the peroneal nerves of rabbits: (1) in normal nerves, and (2) in the proximal stumps of nerves which had been crushed 56–123 days previously. All velocities given in m./sec.

Normal nerves			Proximal stumps		
Animal	Inflexion velocity (m./sec.)	Spike velocity (m./sec.)	Animal	Inflexion velocity (m./sec.)	Spike velocity (m./sec.)
897	71·5	59·1	897	75·6	61·2
921 L	76·7	57·5	927	79·5	62·0
921 R	73·7	56·3	965	66·5	62·7
922	65·4	48·5	966	79·3	74·1
927	61·0	49·1	967	81·7	68·5
961	61·3	50·5	988 L	72·8	66·4
962	75·4	58·6	988 R	77·1	68·3
963	71·9	58·1	989	75·7	66·1
964	65·8	60·3			
965	69·6	66·1			
966	64·1	54·0			
989	66·7	62·4			

Mean inflexion velocity = 68·6 m./sec.
Standard error = 1·5.
Mean spike velocity = 56·7 m./sec.
Standard error = 1·4.

Mean inflexion velocity = 76·0 m./sec.
Standard error = 1·8.
Mean spike velocity = 66·2 m./sec.
Standard error = 1·8.

Comparison of mean velocities:

For inflexion velocity, $t_{18} = 3{\cdot}190$, whence $P = 0{\cdot}01–0{\cdot}001$.
For spike velocity, $t_{18} = 4{\cdot}170$, whence $P =$ less than 0·001.

Comparison of normal and proximal stump velocities. The broken line in Fig. 3 shows the regression line calculated from the pooled data for the fastest fibres in the twelve normal nerves (see Fig. 2), for comparison with that obtained for the proximal stump data. The line for the proximal stumps has a steeper slope, and it therefore appears that the fibres of the proximal stump of crushed nerves, at times between 56 and 123 days after the lesion, conduct faster than the equivalent fibres in normal nerves. From Table 1 the mean inflexion velocity is 7·4 m./sec. faster, while the mean spike velocity in the crushed nerves exceeds that of its normal counterpart by 9·5 m./sec.

To determine whether these differences in velocity were greater than those to be expected as a result of random sampling, a *t*-test was applied to the data of Table 1. In the case of the inflexion velocity, t, for 18 degrees of freedom, had a value of 3·190, which corresponds to a probability of between 0·01 and 0·001 that the two sets of velocities are different samples taken from the same population. The results of this test therefore indicate, in the case of the first inflexion of the action potential, that the proximal stumps of crushed nerves after the above intervals of regeneration do in fact conduct impulses with significantly greater velocity than normal nerves. A similar test, applied to the data for spike velocity, also showed a significant difference in velocity in favour of the proximal stumps (t, for 18 degrees of freedom, $=4{\cdot}17$; $P=$ less than 0·001).

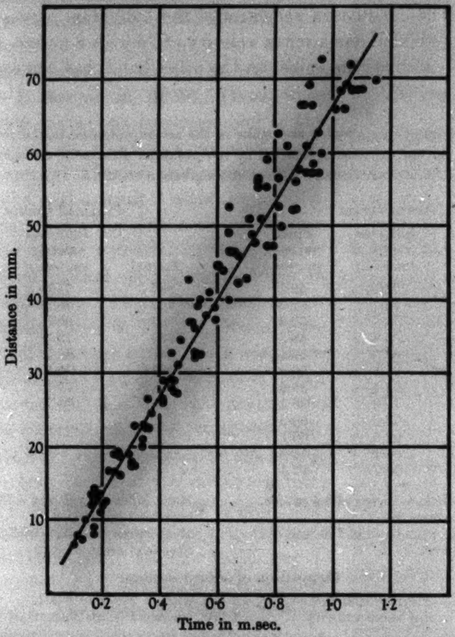

Fig. 2. Graph of conduction distance against time for the inflexion of the action potential in normal nerve. Pooled data from twelve nerves. The line is the calculated regression line for the pooled data.

Conduction rates of regenerating fibres distal to the point of crushing. Action potentials were recorded in twenty-three nerves distal to the point of crushing, and at intervals ranging from 14 to 486 days after operation (Fig. 4). The inflexion and spike velocities obtained are shown in Table 2 and Fig. 5. In the earliest stages, the action potentials were very small (about 10 μV.) and consisted of a single elevation only (Fig. 4). With increasing times of regeneration, the potentials increased in height, and in addition to the main spike there was sometimes also visible a much later, longer lasting, elevation corresponding in position to the *C* wave of normal nerve. Only the 456- and 486-day regenerates, however, showed anything corresponding to the $\gamma\delta$ wave of normal nerve.

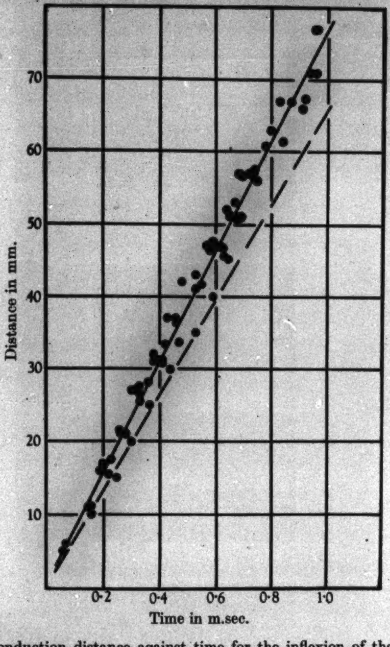

Fig. 3. Graph of conduction distance against time for the inflexion of the action potential in proximal stumps at from 56–123 days after crushing. Pooled data from eight nerves. The solid line is the regression line calculated from these pooled data; the broken line is the regression line for normal nerve given in Fig. 2.

The results given in Table 2 show that the fibres of nerves regenerating after a crush lesion at first conduct impulses slowly, but that with the passage of time the rate increases, finally approximating to that seen in normal nerve. From Fig. 5 it can be seen that the rate of increase of velocity is at first rapid, and then more gradual, the increment of velocity between 146 and 486 days

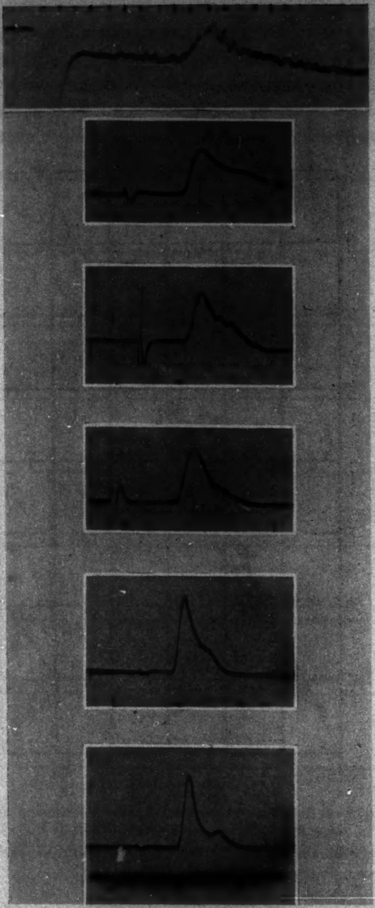

Fig. 4. Action potentials obtained from the distal stumps of regenerating nerves after:

(1) 15 days: conduction distance 69 mm.
(2) 57 ,, ,, ,, 39 ,,
(3) 72 ,, ,, ,, 75 ,,
(4) 82 ,, ,, ,, 70 ,,
(5) 456 ,, ,, ,, 68 ,,
(6) 486 ,, ,, ,, 86 ,,

Time marks = 1/500 sec.

being less than that observed during the first 100 days of regeneration. Unfortunately the data are at present incomplete, lacking observations between 146 and 456 days of regeneration, and it is thus impossible to give precise form to the curve of velocity against time of regeneration. However, in a general way the curve resembles, though it does not closely parallel, the curve of diameter increase with time (Fig. 6).

TABLE 2. Conduction velocities distal to the point of crushing in regenerating rabbit peroneal nerves

Animal	Days of regeneration	Inflexion velocity (m./sec.)	Spike velocity (m./sec.)
340	14	12·8	10·5
(distally)		0·7	0·6
330	15	9·2	—
(distally)		3·7	2·3
329	26	20·6	15·9
(distally)		15·1	12·0
922	55	25·6	22·4
897	56	27·8	25·2
898	57	38·3	33·0
899	57	36·9	36·3
961	60	35·8	32·4
962	63	32·8	27·4
963	63	33·4	31·6
964	67	35·1	33·8
861	72	46·1	35·6
872	82	50·8	43·7
965	114	50·5	44·2
967	114	48·7	46·2
966	115	53·0	41·4
989	123	45·5	43·5
879 R	146	48·7	42·6
879 L	146	48·1	41·2
990 R	456	66·5	54·2
990 L	456	65·9	53·5
948 R	486	61·5	51·8
948 L	486	59·2	51·8

Apart from the 14-, 15- and 26-day animals the conduction rates were measured in the way already described. In these three animals, however, the rates were measured on the nerve in situ, with the whole animal enclosed in a warm, humid, chamber. It was thought that the excision, soaking and setting up in the chamber for excised nerve might abolish conduction in the delicate fibres responsible for the slow conducting waves found in these nerves.

A similar curve of conduction velocity against time for regeneration after suture is given by Berry *et al.* (1944). It resembles the present one in that the increase in velocity with time of regeneration is at first rapid, and then later tails off. However, these authors found that even after 466 days of regeneration the conduction rate was still abnormal, falling short of the normal velocity by about 20%. The data from the four nerves at 456 and 486 days are shown plotted on the same graph in Fig. 7. For the four nerves the inflexion had a mean velocity of 63·3 ± 2·5 m./sec. (S.E. of mean of four observations) while the mean spike velocity was 52·8 ± 2·4 m./sec. (4). Comparing these velocities with those obtained for normal nerve by means of a *t*-test it was found that, taking $P = 0{\cdot}05$ as the conventional level of significance, neither the inflexion nor the spike velocity differed significantly from its normal counterpart. For the inflexion velocity, t, for 14 degrees of freedom, $= 1{\cdot}777$, and therefore $P \simeq 0{\cdot}1$; while for the spike velocity $t = 1{\cdot}404$ and $P = 0{\cdot}1$–$0{\cdot}2$. This, combined with the observation that the form of the action potential in these late regenerates is identical with the normal, showing $\alpha\beta$, $\gamma\delta$, and C waves, is strong

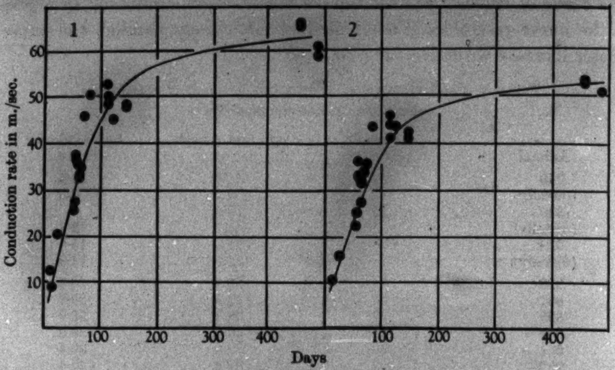

Fig. 5. Graphs of (1) inflexion, and (2) spike velocity in the distal stump against time of regeneration. The curves were drawn by eye.

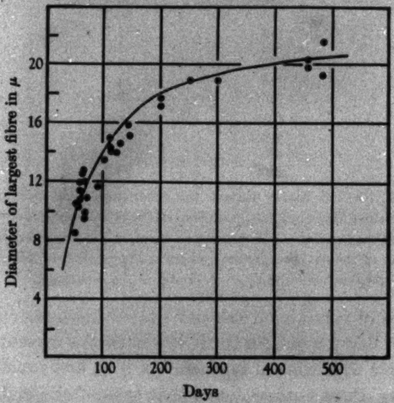

Fig. 6. Graph of the diameters of the largest fibres found in the distal stump against time of regeneration. The extra points in this graph (cf. Fig. 5) were inserted from the data of Gutmann & Sanders (1943). The curve was drawn by eye.

evidence that the four nerves measured had indeed returned to the normal state as regards conduction velocity. Yet the differences from normality in the case of both inflexion and spike velocities came close to significance, and had further observations been available at these times, it is possible that the velocities might be found to be still slightly below normal. All our experiments,

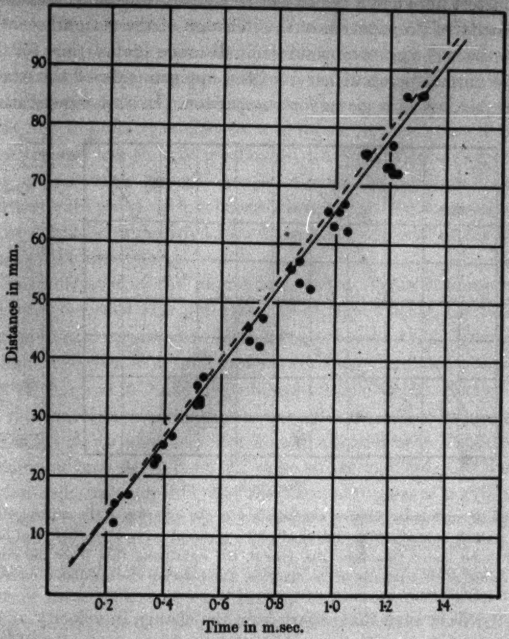

Fig. 7. Graph of conduction distance against time for the inflexion of the action potential in the distal stump after 456 and 486 days of regeneration. Pooled data from four nerves. The solid line is the regression line calculated from these pooled data. The dotted line is the regression line for normal nerve given in Fig. 2.

however, were made upon crushed nerves, while Berry *et al.* (1944) used nerves which had been cut and sutured. It has already been shown (Gutmann & Sanders, 1943) that, after crushing, a nerve becomes completely reconstituted after only 300 days of regeneration, while as late as a year after suture the fibre pattern of the nerve is far from being restored. The results of the present experiments, therefore, emphasize the distinction between regeneration following nerve crushing, and the repair which follows nerve suture. Regeneration after a simple crush injury is the more complete.

In all, except the 456- and 486-day animals, the conduction velocity changed sharply at the point of crushing, the fibres distal to the lesion conducting less fast than those in the proximal stump. Fig. 8 shows a plot of conduction distance against time in the case of a nerve which had been allowed to regenerate for 56 days after it had been crushed (animal 897). Proximal to the lesion the fastest fibre had a velocity of 75·6 m./sec.; distal to the site of crushing the velocity of propagation of the inflexion of the action potential fell to only 27·8 m./sec. The graph of conduction distance against time for the fastest fibre in the normal peroneal nerve of the opposite side of the same animal ($V = 71{\cdot}5$ m./sec.) is also given for comparison. In this regenerating nerve, however, it will be seen that, apart from the change in velocity at the site of crushing, no change in velocity could be detected on passing distally within the distal stump itself. The distal stump was also found to conduct at a constant rate throughout its length in all the other regenerating nerves except those measured at 14, 15 and 26 days after operation. In animal 344 (14 days regeneration time) the conduction rate just distal to the crush was 12·8 m./sec., while lower down the stump it had fallen to 0·7 m./sec., a velocity in the range found for *C* fibres in normal nerve. *C* fibres are unmyelinated, and it was probable that in this region of the stump the impulse was travelling in non-myelinated extensions of the regenerating fibres. Indeed, histological examination of the region of the nerve from which this low rate was recorded, showed that it contained only very fine unmyelinated fibres. In animal 330, measured at 15 days after crushing, the inflexion velocity just below the lesion was

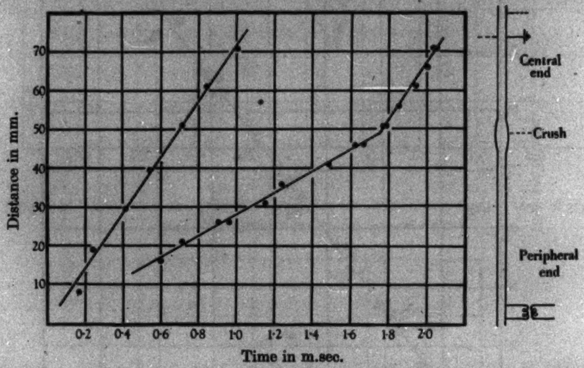

Fig. 8. Graph of conduction distance against time for the inflexion of the action potential in the case of a single animal (897), 56 days after the peroneal nerve of one side had been crushed. The left-hand line is that from the normal, the right-hand that from the crushed nerve. Arrangement of electrodes as in the diagram. The lines are the calculated regression lines.

9·2 m./sec., while further distally it was only 3·7 m./sec. This velocity is at the extreme lower limit of the range of normal myelinated fibres (Grundfest, 1940). The third animal (26 days: animal 329) had fibres which conducted at 20·6 m./sec. just below the lesion. Lower down the stump the velocity was 15·1 m./sec. The action potentials in all three cases were obtained when the stimulating electrodes were placed at the upper end of the proximal stump, and the recording electrodes moved to various points distal to the site of crushing. We can therefore be certain that we are here concerned with impulses that have been propagated from fast-conducting fibres in the proximal stump into slow-conducting extensions of the same fibres. In the case of animal 344 the impulse travelled from a region in which the fibres were of large diameter and less thickly myelinated, and finally into a stretch of fibre which did not possess a stainable myelin sheath. In the second case (animal 330) the fibres were probably myelinated throughout, but the results show that the impulse could be propagated into a tapering extension of the presumably large fibres above the lesion.

In the case of most of the nerves recorded in Table 2 whose conduction velocities were recorded after 56–146 days of regeneration, the rising limb of the action potential appeared to have a steeper slope at all distances of conduction than those seen in normal nerve at corresponding conduction distances. The effect of this was to make the conduction time to the spike of the action potential shorter relative to the time of the inflexion, and to bring the inflexion and spike rates closer together. For nine of the nerves in Table 2 there is a corresponding normal nerve from the same animal in Table 1. Comparison of the inflexion-spike velocity differences in these nine pairs of nerves shows that the set of fibres between those responsible for the inflexion and the peak of the $\alpha\beta$ wave in the normal nerve cover a greater range of velocities than a similar set of fibres in distal stumps, at any rate for the first 67 days of regeneration. The fibres in these regenerates are thus more homogeneous than the normal as regards conduction rate which is to be expected since the fibres in such nerves cover a smaller range of diameters than in normal nerves. Since neither the inflexion nor the spike velocities of the normal and late regenerates (456 and 486 days) differ significantly from one another, it is obvious that at the end of regeneration the normal range of velocities is restored.

Functional recovery and return of conduction velocity

Apart from animals which were killed at 14, 15 and 26 days after crushing, all the animals showed complete functional recovery, as estimated by the return of toe-spreading of normal amplitude (Gutmann, 1942), at the time when the nerves were excised for conduction rate measurement. From Table 2 it can be seen that, except for the late regenerates in which the normal conduction velocity had been completely restored, none of these nerves had a normal conduction rate. Animal 922, for example, in which the distal stump conducted impulses at a rate of only 25·6 m./sec. as compared with 68·6 m./sec. in the normal, nevertheless showed a spreading reflex of normal amplitude. From these results we can conclude that a nerve whose constituent fibres conduct at an abnormally slow rate can nevertheless produce at the end-plates a pattern of impulses sufficiently like the normal to elicit the contractions of the muscles seen in a normal spreading reflex. However, the reflex spreading of the toes is produced by the co-ordinated contraction of a small group of muscles situated close together at about the same distance from the spinal cord. Thus, although the conduction rate of the distal stump of the nerve is abnormally low, impulses can presumably arrive at the end-plates of the different muscles of the group without their time relations being greatly disturbed. Provided sufficient muscle fibres have been re-innervated toe-spreading of normal amplitude will be possible in spite of a slow conduction rate. The total reaction time will probably be increased, but this is not detectable under the conditions of testing used.

Relation of conduction velocity to myelin sheath thickness

Measurements of the total diameter, the axon diameter, and the myelin thickness were made on both normal and regenerating nerves in the way already described. The largest fibres in the normal nerves had a mean total diameter of $20{\cdot}4 \pm 0{\cdot}35\,\mu$ (S.E. of mean of 11 observations), an axon diameter of $15{\cdot}4 \pm 0{\cdot}34\,\mu$ (S.E. of mean of 11 observations), and a mean double thickness of the myelin of $5{\cdot}0 \pm 0{\cdot}08\,\mu$ (11). In the eight proximal stumps whose conduction rates were measured after 56–146 days of regeneration, the largest fibre had a mean total diameter of $19{\cdot}2 \pm 0{\cdot}34\,\mu$ (8). The mean axon diameter of these fibres was $13{\cdot}3 \pm 0{\cdot}47\,\mu$ (8), while their myelin sheaths had a mean double thickness of $5{\cdot}8 \pm 0{\cdot}13\,\mu$ (8). The dimensions of the largest fibres in these nerves were compared with those of normal nerves by means of a t-test. For total diameter, axon diameter, and sheath thickness t had a value corresponding to a probability of $< 0{\cdot}05$, indicating a significant difference between the two types of nerve. Therefore the largest fibres in the proximal stumps of nerves which had been regenerating for 56–146 days had smaller axon and total diameters, but thicker myelin sheaths, than normal nerves (Sanders, 1946). This difference is also shown by the photomicrographs of normal and proximal stump fibres given in Fig. 9.

Were the conduction velocity related solely to either the axon or the total diameter (Gasser & Grundfest, 1939; Hursh, 1939) these proximal stump fibres should conduct more slowly than normal nerves, since they have smaller axon and total diameters. Actually, as has been shown on p. 158, they conduct significantly faster than the corresponding normal fibres. The only positive change with which this increase in velocity can be correlated is the increase in myelin sheath thickness which occurs during the transformation of normal fibres into the type seen in these proximal stumps. These results therefore suggest that conduction velocity depends, in the first instance, upon myelin sheath thickness, fibres with a thick sheath conducting faster than fibres of similar, or even greater, diameter but with thinner sheaths.

Further evidence of the dependence of conduction velocity upon sheath thickness was obtained from the data relating to distal stumps and normal nerves. Sanders (1946) has shown that the myelin sheaths of fibres in the distal stumps of regenerating nerves are at first thicker than the sheaths of fibres of corresponding diameter in normal nerve. On the assumption that myelin thickness controls conduction velocity, these distal stump fibres should conduct faster than fibres of the same axon diameter in normal nerves. Although a group of normal fibres with which they may be directly compared cannot be isolated at present, it has been possible to obtain further information in a different way. In Fig. 10 the inflexion velocity of each nerve used, whether normal, proximal, or distal stump, is shown plotted against (1) the total diameter, (2) the axon diameter, and (3) twice the myelin thickness (i.e. total diameter minus axon diameter) of the largest fibre present. The experimental points on inspection show less scatter about the calculated regression line in the case of sheath thickness than in either of the other cases. However, this apparent reduction of scatter may be misleading, since the regression line of velocity on myelin thickness has a somewhat steeper slope than the regression on total or axon diameter. Partial regression analysis of the data was therefore undertaken, and the details are given in Table 3. The total sum of squares of the velocity as found in these data was 10,162 m./sec.^{2}. The multiple regression accounted for 8879 m./sec.^{2} of this, leaving an error sum of squares of 1283 with 35 degrees of freedom and therefore an error mean square of 36·66. The regression on axon diameter alone gave a sum of squares of 6197; the difference between it and the multiple regression had a mean square of

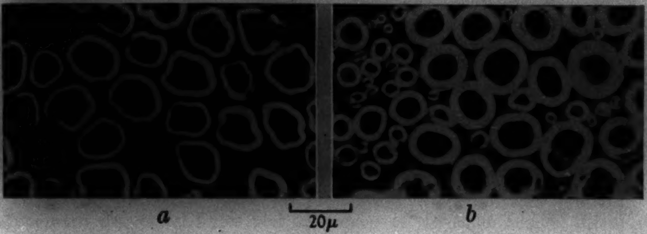

Fig. 9. Photomicrographs, made by direct projection on to bromide paper, of (*a*) fibres from a normal nerve, and (*b*) fibres of corresponding diameter from a proximal stump after 114 days of regeneration. Note thickened myelin sheaths in proximal stump fibres.

2682, which was highly significant compared with the mean square for error. Similarly, the regression on total diameter accounted for a sum of squares of 8359, leaving a residue when subtracted from the multiple regression sum of squares of 520 m./sec.^{2}, with one degree of freedom; this difference was again significant when compared with the mean square for error. By contrast the difference between the sum of squares for the regression on myelin thickness alone and that for the multiple regression was only 55 m./sec.^{2}, a value which was not significantly different from the mean square for error. The analysis therefore indicates that whereas the multiple regression accounts for significantly more of the total variance than the regressions on total diameter or axon diameter, practically the whole of the sum of squares of the multiple regression can be accounted for by considering the regression on myelin thickness alone. The present data from normal and regenerating nerves therefore

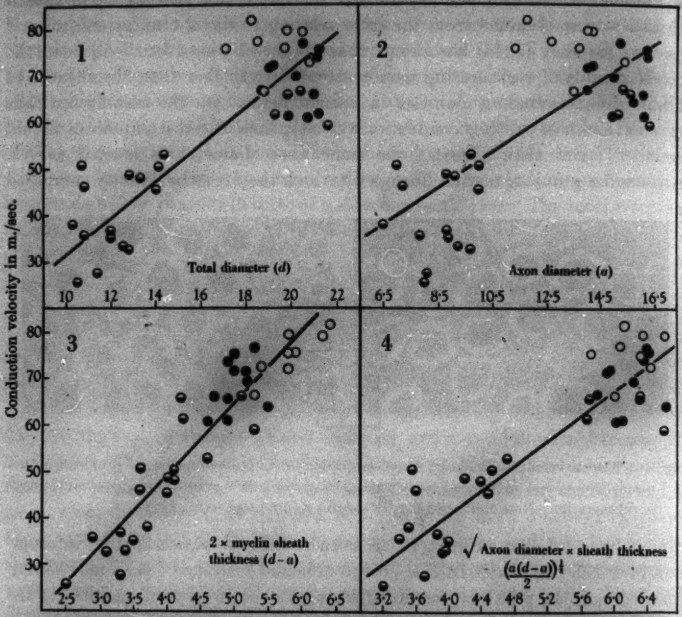

Fig. 10. Graph of the conduction velocity of the inflexion of the action potential against (1) total diameter d, (2) axon diameter a, (3) 2 × myelin sheath thickness, $d-a$, and (4) $(a(d-a)/2)^{\frac{1}{2}}$, for the largest fibre in each nerve. The lines are the calculated regression lines. ● = normal nerves; ○ = proximal stumps; ◒ = distal stumps. The data on which this figure is based are given in an appendix to this paper.

indicate that there is a closer correlation between conduction velocity and sheath thickness than between conduction velocity and axon or total diameter. A further point of interest is that the residual sum of squares used as a measure of error (1283; Table 3) is about 12·6% of the total, which is what would be expected on account of the known sources of non-systematic error listed on p. 155.

TABLE 3. Partial regression analysis of the data of Fig. 10

Item	Degrees of freedom	Sum of squares (m./sec.^{2})	Mean square	Variance ratio	Probability
Regression on axon diam. only	1	6197			
Difference from multiple regression	1	2682	2682	73·16	<0·001
Regression on total diam. only	1	8359			
Difference from multiple regression	1	520	520	14·18	0·001
Regression on myelin thickness only	1	8824			
Difference from multiple regression	1	55	55	1·50	>0·20
Multiple regression	2	8879			
Error	35	1283	36·66		
Total	37	10162			

Conduction velocity and internodal distance

In the case of the four distal stumps which had been allowed to regenerate for 456 and 486 days (Table 2), portions were fixed and teased by our colleague Mr A. D. Vizoso to demonstrate the nodes of Ranvier. Whereas in normal nerve large fibres have longer internodes than fibres of smaller diameter, in these regenerated nerves all fibres had internodes of the same length, the actual length being in the case of large fibres much shorter than that found normally. Fig. 11 shows a large fibre taken from one of the 486-day regenerates compared with a fibre of similar diameter taken from a normal peroneal nerve. The regenerated fibre has four internodes in the length occupied by two internodes in the normal fibre. Such differences were a constant feature of all the fibres examined.

Were conduction velocity primarily a function of internodal distance, we should expect such fibres with short internodes to conduct impulses more slowly than normal fibres. In fact, as already stated (p. 162), neither the inflexion nor the spike velocities differed significantly from normal. This must indicate that conduction rate can vary independently of internodal distance. Moreover, the myelin sheath thicknesses of the late regenerated fibres resembled those of normal nerves.

DISCUSSION

From the results of these experiments we may conclude that within the homogeneous group of fibres provided by a mixed nerve of a single mammalian species, there is a direct dependence of conduction velocity upon myelin-sheath thickness. This conclusion does not invalidate the relation between velocity and total diameter previously found by many workers (Hursh, 1939), because, as already stated, total diameter and myelin thickness are themselves interrelated. The relation between myelin thickness and the total fibre diameter is however only approximately a linear one (Sanders, 1946), and this may account for the inadequacy of reconstructions of the compound action potential based upon the relation $v = kd$ (Gasser & Grundfest, 1939). These authors, however, found that adequate reconstructions could be made on the assumption that velocity varies linearly with axon diameter. Now it has been found that over the range of fibre diameters in which the observations of Gasser & Grundfest (1939) were made (1–13 μ) there is an approximately linear relationship between myelin thickness and axon diameter, the graph connecting the two quantities having only a slight upward curvature. A similar slight upward convexity is seen in the empirical axon diameter-velocity curve derived by Gasser & Grundfest (1939) from their reconstructions (their Fig. 13*b*). Thus there is reason to believe that the assumption $v = k \times$ *myelin sheath thickness* would also give adequate reconstructions of the action potential.

It is not suggested, however, that sheath thickness is the only variable determining conduction rate. Pumphrey & Young (1938) found that in cephalopod fibres, where there is no 'myelin' sheath in the accepted sense, the axons being surrounded by a thin sheath containing a relatively small proportion of lipids, the velocity varies approximately as the square root of the axon diameter. A similar relationship for this type of fibre has been obtained by Offner, Weinberg & Young (1940) from a mathematical treatment of the core-conductor theory of nerve conduction. Hence it is probable that other dimensions of the fibre in addition to myelin thickness may also play a part in controlling conduction velocity in myelinated fibres. Moreover, while myelin sheath thickness may be the main factor determining conduction rate in a homogeneous group of myelinated fibres, such as those provided by a single peripheral nerve of one mammalian species, it is

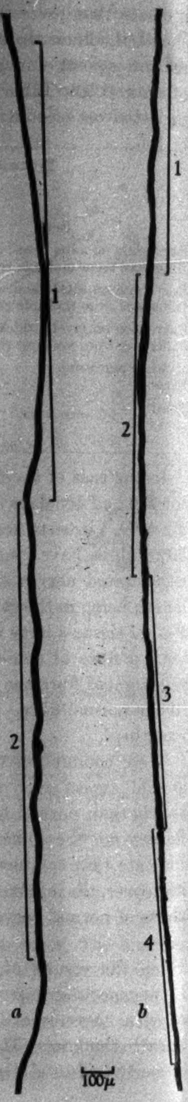

Fig. 11. Photomicrographs of comparable fibres teased from (*a*) normal nerve, and (*b*) a distal stump after 486 days of regeneration. Successive internodes are marked by brackets and the figures 1, 2, etc.

probably necessary to take other factors, such as variations in sheath composition, into account, when interspecific comparisons are made. A reliable estimate of sheath composition in terms of the relative proportions of lipid and protein present is provided by the sheath birefringence (Schmitt & Bear, 1939). The importance of sheath composition with regard to conduction velocity is shown by the work of Taylor (1942) and Taylor & Werndle (1943). These authors compared the fibre diameters and sheath birefringence of a structurally very diverse set of fibres (squid giant: earthworm giant: shrimp giant: frog sciatic: cat saphenous and catfish Muller fibres), all of which conducted impulses at a speed of approximately 25 m./sec. When the fibres were arranged in descending order of diameter, it was found that they were then in ascending order of sheath birefringence, the fibres whose sheaths had the strongest positive birefringence, and accordingly contained the most lipid, being those with the smallest diameters, and vice versa. Whether similar differences in sheath composition cause the nerves of different mammals to conduct at different rates is not known, since data on both conduction rate and birefringence are not available for a sufficiently diverse range of species. That such is the case, however, is suggested by the fact that while the largest fibres in the peroneal nerves of both cat and rabbit have the same diameter (20μ) and the same myelin thickness ($2{\cdot}5\mu$), the cat's nerve conducts at 108–111 m./sec. (Hursh, 1939), whereas the maximum velocity attained in the rabbit's nerve is only in the region of 69 m./sec.

The precise mechanism whereby a change in myelin thickness alters the speed at which the nerve impulse travels is at present largely a matter for speculation. Recent work on large 'unmyelinated' invertebrate fibres has established that in such fibres nervous transmission is brought about by local electric circuits, and it is improbable that the mechanism in myelinated fibres should be fundamentally different. On local circuit theory conduction velocity will be directly proportional to the rate at which the local circuits spread along the fibre. This latter rate is determined by the expression $t = (\text{axon diameter} \times \text{sheath thickness})^{-\frac{1}{2}}$, where t is the time taken by the local circuits to spread a given distance. Hence velocity should vary as $(\text{axon diameter} \times \text{myelin thickness})^{\frac{1}{2}}$. To test whether the present data are in accord with such a hypothesis, Fig. 10, 4 was constructed. In this figure the velocities for all the different nerves used are shown each plotted against the appropriate value of $(\text{axon diameter} \times \text{myelin thickness})^{\frac{1}{2}}$, with the calculated regression line drawn to fit the data. On inspection Fig. 10, 4 does not show a markedly closer correlation between conduction velocity and $(\text{axon diameter} \times \text{myelin thickness})^{\frac{1}{2}}$ than that already seen between velocity and myelin thickness by itself (Fig. 10, 3). Moreover, the data are insufficient to show whether a linear regression or one of higher order should be used to express the relationship plotted in Fig. 10, 4. All that can be said is that the above hypothesis is not excluded by the present data.

On the other hand, the theoretical treatment developed by Offner *et al.* (1940) has led these authors to conclude that velocity is related to some function of the nodal widths and internodal distances, which is contrary to the results reported in the present paper. This theory assumes that the myelin forms an insulating layer over the surface of the fibre, and that the impulse is forced to jump from node to node without causing excitation of the intervening portions of the fibre. Yet in the central nervous system the fibres have no nodes, and conduction velocity is just as high. The whole question of the part played by variations in the dimensions and composition of the myelin sheath is in need of further investigation before an adequate theory of conduction in myelinated fibres can be developed.

SUMMARY

1. Measurements have been made of conduction velocities in rabbits' peroneal nerves regenerating after a crush lesion: (*a*) in the proximal stump after 56–486 days of regeneration; (*b*) in the distal stump after 14–486 days of regeneration.

2. Proximal to the lesion, after 56–146 days of regeneration, the largest fibres conduct at about 7·5 m./sec. (11%) faster than the corresponding fibres in normal nerves. Such fibres have thicker myelin sheaths than the corresponding normal fibres.

3. In the earliest days of regeneration, fibres distal to the lesion conduct at relatively low velocities (<10 m./sec.), and show a diminution in velocity on passing distally within the stump. With increasing time of regeneration, the conduction rate increases, at first rapidly, and thereafter more slowly, until after 456 and 486 days of regeneration the rates recorded do not differ significantly from those of normal nerves.

4. In nerves which have been allowed to regenerate for 456 and 486 days the nodes of Ranvier on the largest fibres are more closely spaced than on fibres of corresponding diameter in normal nerve, although the two types of fibre conduct at similar rates.

5. As a result of these experiments it is concluded that, in the peroneal nerve of the rabbit: (*a*) conduction velocity has a relation to myelin sheath thickness, and is (*b*) independent of internodal distance.

APPENDIX

In the accompanying table the data for inflexion velocity (V), the total diameter (d), the axon diameter (a), and double myelin thickness ($d-a$) of the largest fibre present are summarized for each of the nerves in which it was possible to measure them all. The first column gives the number of the animal together with the limb, right (R), or left (L), from which the particular nerve was taken: the second column indicates whether the particular nerve measured was normal (N), proximal (P), or distal (D) stump; subsequent columns give the values found for V, d, a, and $d-a$ in each case.

Animal	Type of nerve	V	d	a	$(d-a)$
922 *R*	*D*	25·6	10·5	8·0	2·5
897 *L*	*D*	27·8	11·4	8·1	3·3
962 *L*	*D*	32·8	12·8	9·7	3·1
963 *R*	*D*	33·4	12·6	9·3	3·4
964 *R*	*D*	35·1	12·4	8·9	3·5
961 *R*	*D*	35·8	10·7	7·8	2·9
899 *L*	*D*	36·9	12·1	8·8	3·3
898 *L*	*D*	38·3	10·2	6·5	3·7
989 *R*	*D*	45·5	14·0	10·0	4.0
861 *R*	*D*	46·1	10·8	7·2	3·6
879 *L*	*D*	48·1	13·3	9·2	4·1
879 *R*	*D*	48·7	12·8	8·8	4·0
965 *R*	*D*	50·5	14·1	10·0	4·1
872 *L*	*D*	50·8	10·6	7·0	3·6
966 *L*	*D*	53·0	14·3	9·7	4·6
948 *L*	*D+P*	59·2	21·6	16·3	5·3
927 *L*	*N*	61·0	20·7	16·1	4·6
961 *L*	*N*	61·3	19·9	14·9	5·0
948 *R*	*D+P*	61·5	19·3	15·1	4·2
966 *L*	*N*	64·1	21·2	15·7	5·5
922 *L*	*N*	65·4	22·7	17·7	5·0
990 *L*	*D+P*	65·9	19·8	15·6	4·2
990 *R*	*D+P*	66·5	20·4	15·3	5·1
965 *R*	*P*	66·5	18·8	13·5	5·3
989 *L*	*N*	66·7	18·7	14·0	4·7
965 *L*	*N*	69·6	20·2	15·0	5·2
897 *R*	*N*	71·5	19·0	14·0	5·0
963 *L*	*N*	71·9	19·2	14·2	5·0
988 *L*	*P*	72·8	20·8	15·4	5·4
921 *R*	*N*	73·7	21·1	16·2	4·9
962 *R*	*N*	75·4	21·2	16·2	5·0
897 *L*	*P*	75·6	17·1	11·3	5·8
989 *R*	*P*	75·7	19·7	13·8	5·9
921 *L*	*N*	76·7	20·5	15·2	5·3
988 *R*	*P*	77·1	18·5	12·7	5·8
966 *R*	*P*	79·3	20·5	14·2	6·3
927 *R*	*P*	79·5	19·8	14·0	5·8
967 *R*	*P*	81·7	18·2	11·8	6·4

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THE PRODUCTION AND REMOVAL OF OEDEMA FLUID IN THE LUNG AFTER EXPOSURE TO CARBONYL CHLORIDE (PHOSGENE)

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Many investigations into the effects of exposure to irritant gases were made during the 1914–18 war (Dunn, 1918; *The Official History of the War*, 1923; Laquer & Magnus, 1921; The Medical Department of the United States Army in the World War, *Medical Aspects of Gas Warfare*, 1926; Underhill, 1920; Winternitz, 1920). Further experiments have been undertaken in an endeavour to clarify the phenomena involved in the production and removal of oedema fluid from the lungs of animals exposed to phosgene.

METHODS

Blood changes resulting from exposure to phosgene were investigated in rabbits, in dogs and in goats. All these animals were unanaesthetized. Blood samples were obtained from the ear veins of rabbits and from the external jugular veins of dogs and of goats. Oedema fluid was collected by inverting the animal immediately after death or when the animal was killed. The fluid was always clear and macroscopically free from blood contamination.

The plasma volume was estimated by the use of Evans Blue dye, the method followed being that described by Courtice (1943). Haemoglobin percentage was determined by the Haldane haemoglobinometer and plasma and oedema fluid proteins by micro-Kjeldahl digestion and Nesslerization (Wong, 1923). Albumin was similarly determined after precipitation of globulin with 22·5% sodium sulphate at 37° C. Non-protein nitrogen was estimated by precipitation of the proteins with trichloroacetic acid, micro-Kjeldahl digestion and Nesslerization.

The lymph flow from the lungs before and after exposure to phosgene was determined in dogs anaesthetized with 'Nembutal'. In the dog the lymph from the lungs and heart drains into the right lymph duct (Drinker, 1942; Warren & Drinker, 1942). To avoid opening the thoracic cavity and the use of artificial respiration, the right lymph duct was cannulated just above the pleura. As the normal composition of heart lymph and lung lymph is approximately the same, and as any increase in flow during pulmonary oedema probably comes from the lungs, but little error is introduced by collecting this mixed lymph and assuming it all to come from the lungs. Clotting of the lymph was prevented by introducing dry heparin into the cannula at intervals. The protein content was determined by the methods used for plasma proteins.

Blood samples were obtained from one femoral artery and the blood pressure was recorded from the other.

The animals were exposed to the phosgene in a static chamber, 10 cu.m. in volume. The concentration of phosgene was 440 mg./cu.m. and the time of exposure varied from 10 to 30 min. The phosgene concentrations were determined by drawing a known volume of air from the chamber into a bubbler containing 15 c.c. of a solution of hexamine in sodium hydroxide. The resulting chloride was estimated by titration with mercuric nitrate solution. The over-all accuracy of the estimation was ±10%.

RESULTS

Blood changes

Rabbits. Twenty-four rabbits were exposed to phosgene in the following groups: four for 15 min., eight for 20 min., eight for 25 min. and four for 30 min. Increased time of exposure caused oedema of greater severity. The mean results of experiments on three rabbits that survived out of a group of four, exposed for 20 min., are shown in Fig. 1. Although these rabbits survived, they all suffered from considerable respiratory distress. The changes in plasma volume were slight and there was but little haemoconcentration. In spite of such little change in the concentration of the blood, the plasma protein level fell and rose again when the animals recovered. Similar results were obtained in the group exposed for 15 min.

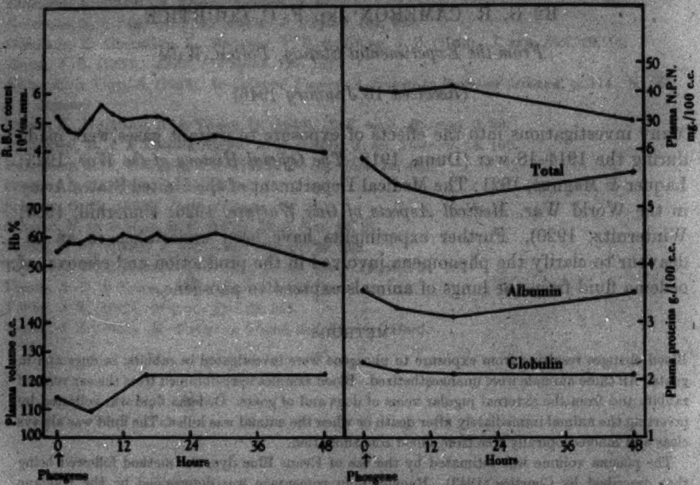

Fig. 1. The effects of exposure of rabbits to 440 mg. phosgene/cu.m. for 20 min. Average of three experiments.

With a longer exposure, 30 min., rabbits usually died quickly with massive pulmonary oedema, and yet showed little or no concentration of the blood. In Table 1 are given the haemoglobin percentages, red cell counts and the lung/heart weight ratio in a typical case. The mean results of twelve experiments in which the animals were exposed for 25 or 30 min. show that the haemoglobin percentage was 66 before exposure and 67 just before death, while the lung/heart weight ratio was 7·6. Death occurred in seven animals from 4 to 7 hr. after exposure and five were killed 6¼ hr. after exposure. Assuming a normal lung/heart weight ratio of 1·8, the average excess fluid in the lungs was 31 c.c. In four of these animals the average plasma volume was 116 c.c. before exposure and 117 c.c. just before death.

TABLE 1. Effects of exposure of rabbits to phosgene, 440 mg./cu.m. for 30 min.

Time		Hb%	R.B.C.	Lungs/heart
Before phosgene		82	5·74	—
After phosgene:	1 hr.	83	5·89	—
	2 hr.	79	5·83	—
	3 hr.	75	5·78	—
	4 hr.	78	5·88	—
	5 hr.	80	5·95	—
	5¼ hr.	Died	—	6·0

It thus appears from these experiments that, in the rabbit, oedema develops rapidly, and the loss of fluid from the blood stream into the lungs is equally rapidly compensated by a withdrawal of tissue fluid into the circulation. As a result of this compensation, the plasma-protein concentration falls considerably. An examination of the proteins in the oedema fluid reveals that plasma protein passes out into the lungs. Table 2 gives the average changes produced in ten rabbits. It is evident that the oedema fluid contains a high protein concentration, lower than the initial plasma-protein level but higher than the final level.

TABLE 2. The proteins, g./100 c.c., of the plasma and of the oedema fluid in the lungs of rabbits about 6 hr. after exposure to phosgene and just before death. Means and S.E. of means of ten experiments

	Before exposure	After exposure
Plasma proteins	5·57±0·12	4·37±0·18
Plasma albumin	3·22±0·04	2·63±0·09
Plasma globulin	2·35±0·12	1·74±0·13
Oedema fluid proteins	—	4·79±0·24
Oedema fluid albumin	—	3·71±0·26
Oedema fluid globulin	—	1·08±0·18

The loss of plasma proteins from the blood stream is approximately balanced by the gain of protein in the oedema fluid. In a group of ten rabbits in which the plasma volume and plasma-protein concentration were measured, the average fall in total circulating plasma protein was 1·3 g. The average volume of oedema fluid at death about 6 hr. after exposure was 32 c.c. containing 1·5 g. protein. As the time during which these changes took place was short, the amount of protein replacement or regeneration was probably not very great. These results, therefore, suggest that plasma protein is lost from the circulation only into the lungs.

If rabbits were kept on a drier diet than normal for a few days before exposure to phosgene, the blood usually concentrated much more as the pulmonary oedema developed. This is shown in Table 3. It seems, therefore, that the rabbit normally has a readily available store of fluid with which to replace the lost plasma, and, if this store is reduced, haemoconcentration occurs as pulmonary oedema develops.

There is also some evidence that haemoconcentration after exposure to phosgene is a protecting mechanism and reduces the risk of death. Ten normal rabbits and ten rabbits that had been on a drier diet than normal for 3 days were exposed together to the same concentration of phosgene for the same time. Eight of the control rabbits died and only three of those that had been on the drier diet.

Table 3. Effect of previous water restriction on haemoconcentration in rabbits after exposure to phosgene

	Haemoglobin %			
	Rabbit 573	Rabbit 574	Rabbit 575	Rabbit 576
Start of water restriction				
22. iii. 41	53	60	58	52
24. iii. 41	72	78	73	68
25. iii. 41	68	80	75	68
26. iii. 41	72	86	88	70
27. iii. 41	70	83	88	70
28. iii. 41	74	90	88	68
Exposed to phosgene				
2 hr. after	63	88	86	64
4 hr. after	80	88	82	58
6 hr. after	76	80	96	80
24 hr. after	85	110	106	100
30 hr. after	82	110	108	93

Dogs. The behaviour of dogs after exposure to phosgene is in some respects different from that of rabbits. The mean results of experiments on nine dogs that survived out of a series of twelve are shown in Fig. 2. The plasma volume has fallen considerably 12 hr. after exposure, and is at about the same level 24 hr. after exposure. It then gradually rises to normal.

Associated with this fall in plasma volume is a rise in haemoglobin percentage and in red cell count. The onset of haemoconcentration and of symptoms is relatively slow. Three hours after exposure the blood is slightly more dilute, but 6 hr. after exposure haemoconcentration begins and rapidly develops. Up to 6 hr. there are no obvious respiratory symptoms, but at 12 hr. the animal shows signs of respiratory distress and cyanosis. These signs continue until about 36 hr. after exposure. During this time, from 12 to 36 hr. after exposure, the blood is very concentrated, the gums and tongue are cyanosed, the ears and extremities are cold and the peripheral veins are collapsed. The animal, therefore, appears to be in a state of shock.

The levels of plasma protein fall and reach a minimum 24 hr. after exposure. They then gradually rise to normal as the plasma volume increases. The fall in the protein is due to a fall in both the albumin and globulin fractions, as in the case of the rabbit. The oedema fluid protein at death in fatal cases, 16–24 hr. after exposure, is approximately equal to that in the plasma, Table 4.

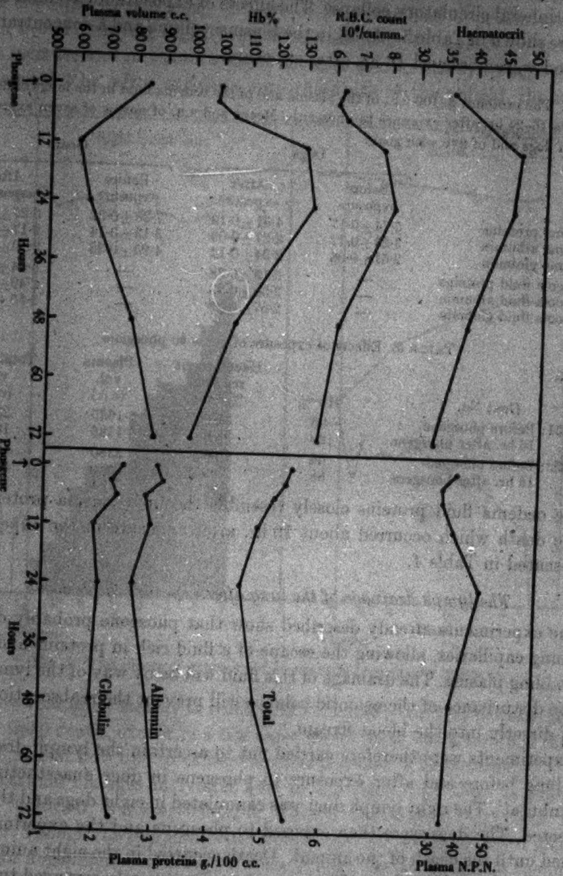

Fig. 2. The effects of exposure of dogs to 440 mg. phosgene/cu.m. for 10 min. Average of nine experiments.

Goats. Goats behave in a similar manner to dogs. Pulmonary oedema is relatively gradual in onset, compared with the rapid rate of development in rabbits, and is accompanied by a considerable degree of haemoconcentration with peripheral circulatory collapse. The effects of exposure for 25 min. on two goats are shown in Table 5. A fall in the plasma volume and a concentration of the blood were observed 16 hr. after exposure.

TABLE 4. The proteins, g./100 c.c., of the plasma and of the oedema fluid in the lungs of dogs and goats 16–24 hr. after exposure to phosgene. Means and S.E. of means of seven experiments with dogs and of five with goats

	Dogs		Goats	
	Before exposure	After exposure	Before exposure	After exposure
Plasma proteins	5·74±0·17	4·51±0·19	7·38±0·32	5·27±0·41
Plasma albumin	3·23±0·12	2·27±0·09	3·13±0·14	2·17±0·18
Plasma globulin	2·51±0·08	2·24±0·12	4·25±0·36	3·10±0·28
Oedema fluid proteins	—	5·15±0·38	—	5·04±0·39
Oedema fluid albumin	—	3·08±0·33	—	2·49±0·25
Oedema fluid globulin	—	2·07±0·29	—	2·55±0·25

TABLE 5. Effects of exposure of goats to phosgene

Goat No.	Hb %	Haematocrit reading % cells	Plasma vol. (c.c.)	Total blood vol. (c.c.)
251: Before phosgene	96	31·6	1635	2390
16 hr. after phosgene	129	38·6	1185	1930
252: Before phosgene	66	20·6	3380	4260
16 hr. after phosgene	88	28·9	2240	3150

The oedema fluid proteins closely resemble the final plasma proteins just before death which occurred about 16 hr. after exposure in the experiments represented in Table 4.

The lymph drainage of the lung after exposure to phosgene

The experiments already described show that phosgene probably damages the lung capillaries, allowing the escape of a fluid rich in proteins and closely resembling plasma. The drainage of this fluid will be by way of the lymphatics, as the disturbance of the osmotic balance will prevent the reabsorption of the fluid directly into the blood stream.

Experiments were therefore carried out to ascertain the lymph drainage of the lung before and after exposure to phosgene in dogs anaesthetized with 'Nembutal'. The right lymph duct was cannulated in eight dogs and the lymph collected. The dogs were then exposed to phosgene and the experiment continued until the death of the animal. Death occurred in the eight animals from 5½ to 12½ hr. after exposure. The exposure was purposely arranged to produce severe pulmonary oedema fairly quickly. The results in all cases were similar, and a typical experiment is represented in Fig. 3.

The lymph flow. The normal lymph flow from the right lymph duct in a series of seventeen dogs, of an average weight of 21·9 kg., varied from 0·1 to

8·2 c.c./hr. with an average of 2·6 c.c./hr. Considering the amount of blood passing through the lungs and the rich plexuses of lymph capillaries, this flow is not great.

After exposure to phosgene, the rate of flow remained approximately the same for 2–5 hr., varying slightly in different animals. In some cases there was a slight increase and in others a slight decrease during this first stage. In the next stage, 2–5 hr. after exposure until the death of the animal, the rate of lymph flow rose fairly steeply to reach a considerable level. This increase lasted as a rule until death, except in a few cases where there was a terminal falling off in the rate of flow due to exhaustion and decrease in respiration.

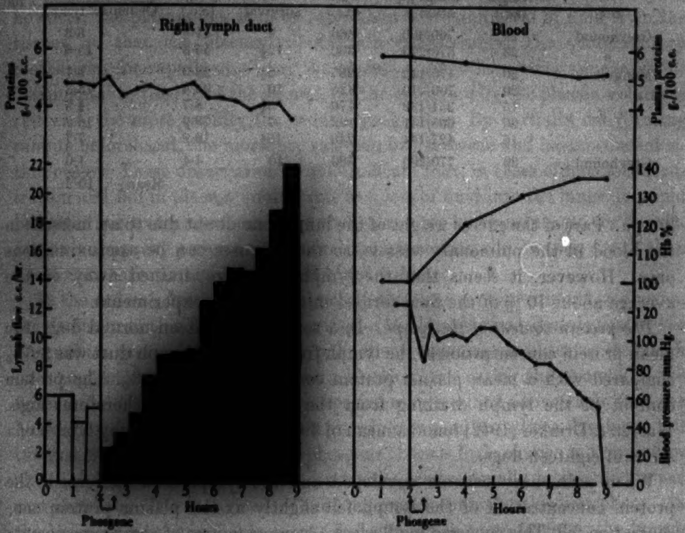

Fig. 3. The effects of exposure of an anaesthetized dog to phosgene.

The maximal changes in lymph flow in the eight dogs were as follows: 1·6–10·6, 0·5–18·0, 0·8–8·4, 5·0–22·0, 0·3–4·2, 5·2–22·0, 0·1–2·4 and 1·2–3·7 c.c./hr. Though the lymph flow before exposure to phosgene varied greatly in different dogs, there was always a considerable increase in flow as the lungs became more oedematous.

The rate of removal of oedema fluid from the lungs can be estimated from the lymph flow. The production of oedema fluid is not so easy to determine, but the measurement of the lung/heart weight ratio will give an approximate indication of the excess amount of fluid in the lungs at the end of the experiment. Calculations have been based on normal lung/heart weight ratios of 1·0 for greyhounds and 1·3 for other dogs. In Table 6 are shown the amounts of oedema fluid in the lungs at death, the time of survival after phosgene and the ratio between the lymph volume and the total volume of oedema fluid plus lymph. Part of the excess weight of the lungs is no doubt due to an increase in the blood in the pulmonary vessels, so these figures can be approximations only. However, it seems that the lymphatics have drained away on the average about 10% of the fluid formed in these acute experiments.

TABLE 6. Volume of oedema fluid formed and of lymph drained away, after exposure of dogs to phosgene

Dog	Weight (kg.)	Lungs g./heart g.	Oedema fluid (c.c.)	Hours of survival	Total lymph (c.c.)	$\frac{\text{Lymph}}{\text{Oedema}+\text{lymph}} \times 100$
Greyhound	29	760/300	460	9	41·4	8·3
,,	23	570/270	300	12¼	55·3	15·6
Mongrel	20	470/142	285	6¼	28·9	9·7
,,	23	700/210	426	10	95·3	18·3
,,	15	351/139	170	5¼	8·7	4·9
,,	20	605/151	409	7	78·9	16·2
,,	16	427/144	240	10¼	19·9	7·7
Greyhound	26	770/290	480	10	4·4	1·0
					Mean	10·2

The protein content of the lymph. In a series of seventeen normal dogs, the mean protein concentration of the lymph from the right lymph duct was 3·6% compared with a mean plasma protein concentration of 5·2%. The protein content of the lymph draining from the normal lung is, therefore, high. Warren & Drinker (1942) found a mean of 3·66% protein in the lung lymph of a series of eighteen dogs.

When pulmonary oedema developed and the lymph flow increased, the protein concentration of the lymph fell slightly as the plasma-protein concentration fell. This occurred in all cases. Average figures of eight experiments showed that the plasma-protein concentration fell from 5·2 to 4·1% just before death, the lymph-protein level fell from 4·0 to 3·3% while the oedema-fluid-protein concentration at death was 5·4%.

Blood changes. During the breathing of phosgene the blood pressure fell, probably due to a vagal reflex slowing the heart. After exposure to phosgene the blood pressure generally rose again somewhat, and then fell as haemoconcentration and anoxia progressed. The haemoconcentration and plasma protein changes in these acute experiments were similar to those already described in unanaesthetized dogs.

DISCUSSION

The primary disturbance in phosgene poisoning appears to be damage to the lung capillaries, causing an increase in their permeability to the plasma proteins. This causes an upset of the osmotic effect of these proteins, and oedema results, as in the case of a thermal burn. If the animal survives, oedema progresses for 24–48 hr. after which the disturbance is righted, and then the excess fluid is gradually drained away.

The effects on the blood volume and circulation are secondary to the local loss of fluid. In the rabbit, the fluid loss into the lungs is rapid; but the withdrawal of fluid from undamaged tissues into the blood is also rapid, with the result that the plasma volume usually falls but little. In the dog and goat, however, the plasma loss is more rapid than the withdrawal of fluid from the tissues, so that the plasma volume falls considerably. The compensatory mechanism for rapidly restoring the plasma volume in rabbits appears to be detrimental to the life of the animal, for the more rapidly the plasma volume is restored, the more rapidly the oedema progresses. By partially dehydrating rabbits beforehand, the mortality rate can be decreased and haemoconcentration occurs. These observations would indicate that, in cases of haemoconcentration and fall in plasma volume due to a loss of fluid into the lungs, it would be better not to try to increase the plasma volume by transfusions, for anoxia, due to pulmonary oedema, is a more important factor in causing death than haemoconcentration and decreased blood volume.

As the oedema fluid has approximately the same protein content as plasma, the drainage of this fluid will be by way of the lymphatics. Histologically, the lymphatics are widely dilated in pulmonary oedema, an observation which once suggested that there might be a blockage preventing the removal of the oedema fluid (Vedder, 1925). The experiments described above show that the lymph flow from the lungs of dogs is greatly increased as oedema develops. For the first 2–5 hr. after exposure to phosgene there is little change in lymph flow although interstitial oedema, as seen histologically, usually begins within the first half-hour. The sponginess of the lung may account for this first phase. As the lungs become more oedematous and expiration more forceful, the lymph flow increases rapidly. It has been calculated that the lymphatics removed about 10% of the oedema fluid formed in the experiments described, where death occurred 6–12 hr. after exposure.

After 24 hr., the production of oedema fluid seems to lessen and the animal recovers. If, however, dogs be killed several days later, when haemoconcentration has disappeared, the lungs may still be oedematous. No experiments have been performed on the lymph drainage in this late period, but it appears that, after the acute increase in lymph flow with the rapid onset of oedema, the flow decreases and the excess fluid in the alveoli is gradually drained away in the course of several days. Experiments dealing with the drainage of fluids from the lungs will be described in a subsequent article (Courtice & Phipps, 1946).

It is difficult to explain the behaviour of the lymph proteins after phosgene poisoning. It was expected that the concentration would not fall but rise, as it ment. Calculations have been based on normal lung/heart weight ratios of 1·0 for greyhounds and 1·3 for other dogs. In Table 6 are shown the amounts of oedema fluid in the lungs at death, the time of survival after phosgene and the ratio between the lymph volume and the total volume of oedema fluid plus lymph. Part of the excess weight of the lungs is no doubt due to an increase in the blood in the pulmonary vessels, so these figures can be approximations only. However, it seems that the lymphatics have drained away on the average about 10% of the fluid formed in these acute experiments.

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It is difficult to explain the behaviour of the lymph proteins after phosgene poisoning. It was expected that the concentration would not fall but rise, as it does in pulmonary oedema resulting from lewisite poisoning (Cameron, Courtice & Short, 1946). Post-mortem examinations were made in all cases and the cannulated duct was traced down to the right tracheo-bronchial node into which flowed the afferents coming from the hilum of the lung. In some cases 1 c.c. of 4% Evans Blue dye was inserted by catheter, passed along the trachea, into the lung and dye appeared in the lymph in increasing concentration. In one case 2 c.c. of 4% dye were injected into the right lung about 30 min. before death when it was seen (from the blood pressure and general condition) that the animal would not live much longer. Dye immediately appeared in the tracheal cannula (the animal had been expelling frothy fluid in the tracheal cannula for about 30 min. beforehand), and in 10 min. dye appeared in the lymph and continued to increase in concentration until death. On the other hand, the blood plasma taken at death contained very little blue dye. There seems little doubt, therefore, that the cannulated duct always drained the lung.

SUMMARY

1. Phosgene poisoning has been investigated in rabbits, dogs, and goats in order to obtain further information regarding the process of oedema formation.

2. In rabbits, pulmonary oedema is often rapid in onset, but there is little or no haemoconcentration or change in plasma volume. If the rabbit lives on a drier diet than normal for a few days before exposure to phosgene, the loss of fluid into the lungs causes a considerable degree of haemoconcentration and the risk of death is lessened.

3. The oedema fluid has about the same protein concentration as the plasma, suggesting an increase in capillary permeability. The loss of protein from the blood is approximately the same as the gain in protein in the oedema fluid in the lungs. This confirms that there is only a local loss of fluid in phosgene poisoning.

4. In dogs and goats exposed to phosgene, considerable haemoconcentration occurs as fluid is lost into the lungs. This is accompanied by a fall in plasma protein concentration, both albumin and globulin being affected. As in the rabbit, the oedema fluid closely resembles plasma in its protein composition.

5. The lymph drainage of the lung in anaesthetized dogs has been investigated before and after exposure to phosgene. As the oedema develops, the lymph flow increases rapidly and reaches a level many times the normal. In spite of this great increase in lymph flow, the lymph collected in these acute experiments, where death occurred 6–12 hr. after exposure, amounted on the average to only 10% of the total oedema fluid in the lungs.

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THE ABSORPTION OF FLUIDS FROM THE LUNGS

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When pulmonary oedema is produced by irritant gases such as phosgene, the oedema fluid is similar to the blood plasma in its protein composition (Cameron & Courtice, 1946). As a result, the removal of this fluid is by way of the lymphatics since the effective osmotic pressure of the plasma proteins is abolished. These authors have shown that, in such cases, there is a considerable increase in the lymph flow from the lungs via the right lymph duct during the acute phase of oedema formation. In animals that survive exposure to phosgene, the oedema fluid is but slowly removed over a period of several days after the cessation of the acute phase. This suggests that, after the acute phase, the lymphatic removal of this fluid is very slow.

The present series of experiments was performed to find the rate of removal from the lungs of water, of 0·9% NaCl solution and of serum after they had been introduced into the lungs by way of the trachea.

METHODS

Dogs and rabbits were used. Dogs were anaesthetized with 'Nembutal', the right lymph duct was cannulated and lymph collected. Water, 0·9% NaCl solution or dog serum was then introduced into the lungs via the trachea and the changes in lymph flow measured. The dogs were then killed and the lung/heart weight ratio determined to estimate the amount of fluid remaining in the lungs.

Rabbits were anaesthetized with 'Nembutal', and then water, 0·9% NaCl solution or rabbit serum was introduced into the trachea. The rabbits were then allowed to recover from the anaesthetic and groups were killed at 1, 4, 24, 48 and 72 hr. afterwards. The lung/heart weight ratios were determined and the amount of fluid remaining in the lungs calculated.

In another series of rabbits, 15 c.c. serum were introduced into the trachea of two groups. Special tracheal cannulae incorporating inspiratory and expiratory valves of minimal resistance and dead space were inserted. In one group a pressure of 4 or 6 cm. water was placed on the expiratory side while in the other group no pressure was used. These experiments were designed to show whether or not breathing against a pressure would increase the absorption of serum from the lungs.

RESULTS

Experiments with dogs. Table 1 shows the effects of the introduction of water, of 0·9% NaCl solution and of serum into the lungs of dogs. In each case the fluid was introduced over a period of about 2 hr. From 4 to 5 hr. after the beginning of the injections, the dogs were killed and the lungs examined. Assuming a normal lung/heart weight ratio of 1·3, the excess fluid in the lungs was calculated. Lymph was collected from the right lymph duct throughout, and the excess over normal determined.

TABLE 1. The absorption of fluids from the lungs of normal dogs anaesthetized with 'Nembutal'

Weight (kg.)	Fluid introduced (c.c.)	Fluid absorbed (c.c.)	Excess lymph flow over normal (c.c.)	Duration of experiment (hr.)
14·5	Water 350	283	4	5
18·6	0·9% NaCl 260	156	10	4
14·1	Serum 162	31	1	5
18·0	Serum 55	−3	5	4

The results indicate that water is rapidly absorbed, and 0·9% NaCl solution relatively rapidly absorbed, directly into the blood capillaries, since the increase in lymph flow is small. Serum is but slowly absorbed.

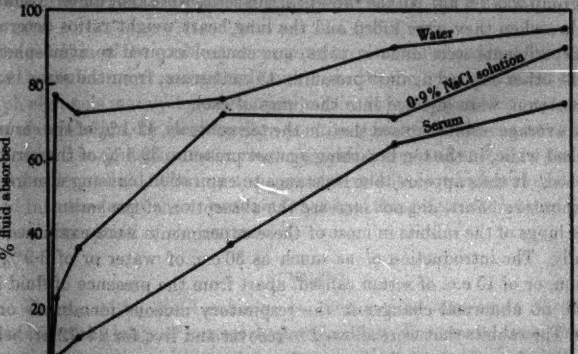

Fig. 1. The absorption of water, of 0·9% NaCl solution and of serum from the lungs of rabbits. 10 c.c. were introduced into each rabbit by way of the trachea. Each point represents the average of five experiments.

Experiments with rabbits. The rabbits were treated in groups of 5. 10 c.c. of fluid were introduced along the trachea into the lungs of each rabbit. Immediately after killing, the lungs were examined and the lung/heart weight ratio determined. From this ratio the excess fluid in the lungs was estimated, assuming a normal ratio of 1·8. (The mean lung/heart weight ratio of ten normal rabbits was 1·76 ± 0·03, S.E. of mean.) The results are plotted in Fig. 1 as the percentage of fluid absorbed. This shows that, with water, nearly 80% was absorbed within 1 hr. and the remainder was then more slowly absorbed. With saline, 23% was absorbed in 1 hr., 36% in 4 hr. and 72% in 24 hr. Serum, on the other hand, was much more slowly absorbed.

In two other groups of rabbits, with 5 in each group, 30 c.c. of water or of 0·9% NaCl solution were introduced into the trachea of each rabbit. Of this, 15 c.c. were introduced first and then, 15 min. later, the final 15 c.c. The animals were killed 30 min. after the beginning of the experiment. From the lung/heart weight ratios it was shown that, on an average, 91·6% of the water was absorbed and 34·5% of the saline.

In a further two groups of rabbits, 20 c.c. of water or of 0·9% NaCl solution were introduced into the lungs of each rabbit and the animals were killed 1 hr. later. At this time 92% of the water and 35·5% of the saline had been absorbed.

In another series of experiments, serum was introduced into the trachea of each of twenty rabbits, two groups of ten. The average weight of the rabbits in each group was 2·8 kg. All the rabbits in this series were kept under 'Nembutal' for 6 hr. when they were killed and the lung/heart weight ratios determined. The experiments were done in pairs, one control expired to atmospheric air and the other expired against pressure. 15 c.c. serum, from the same batch of rabbit serum, were injected into the lungs of each.

The average results showed that, in the ten controls, 42·1% of the serum was absorbed while, in the ten breathing against pressure, 39·4% of the serum was absorbed. It thus appears that resistance to expiration, causing a more forceful expiratory effort, did not increase the absorption of the serum.

The lungs of the rabbits in most of these experiments were examined histologically. The introduction of as much as 30 c.c. of water or of 0·9% NaCl solution, or of 15 c.c. of serum caused, apart from the presence of fluid in the alveoli, no abnormal changes in the respiratory mucous membrane or lung tissue. The rabbits that were allowed to recover and live for 24–72 hr. behaved normally after the initially increased respiration rate returned to normal. After the introduction of water, this recovery was very rapid.

DISCUSSION

These experiments show how rapidly water and 0·9% NaCl solution are absorbed from the normal lungs directly into the blood stream. Very little appears to be absorbed by the lymphatics. Winternitz (1920), while working on the question of pulmonary oedema due to irritant gases, also showed that physiological saline is rapidly taken up by the lungs. The experiments with water are of interest when considering the absorption from the lungs in cases of drowning in fresh water.

The problem of the absorption of lung oedema fluid during the recovery phase of phosgene poisoning, however, is not one of absorption of water or of

0·9% NaCl solution from the lungs. Cameron & Courtice have shown that the oedema fluid is rich in protein and is very similar to plasma. Thus, the experiments in which serum was inserted into the lungs give a much better idea of the rate of removal of oedema fluid after the first acute phase of oedema production is over. It has been seen that serum is very slowly absorbed from the lungs of both dogs and rabbits, and in rabbits it takes about 4 days to remove all the serum introduced. This corresponds to the findings in phosgene poisoning, for, although the animal begins to recover 24–36 hr. after exposure to phosgene, the lungs remain oedematous for about 5 days.

The fact that the lymph does absorb serum at all, even though slowly, has been shown by the introduction of Evans Blue dye into the lungs in some of these experiments. Some of the dye is soon absorbed by the lymphatics and appears in the right lymph duct whereas the plasma contains but little. This dye combines with albumin (Rawson, 1943) and is probably absorbed into the lymphatics combined with protein. It seems, therefore, that the proteins of the serum introduced into the lungs are absorbed by the lymphatics, but at a slow rate.

The solution of the problem of speeding up removal of oedema fluid from the lungs depends on increasing the lymph flow. As the lymph is driven along the lymph channels mainly by mechanical means, it was hoped that increasing the force of expiration, by introducing a positive pressure on the expiratory side of the tracheal cannula, would increase the lymph flow and so hasten the absorption of oedema fluid. The experiments with rabbits in which serum was introduced into the lungs show, however, that breathing against pressure does not increase absorption of the serum.

SUMMARY

1. Water, 0·9% NaCl solution or serum were introduced through the trachea into the lungs of normal dogs and rabbits. The rate of disappearance of these fluids was then estimated.

2. In dogs, the lymph flow from the right lymph duct was measured. Both water and the saline solution passed rapidly from the lungs directly into the blood stream with very little increase in the lymph flow. Serum, on the other hand, was slowly absorbed, probably only by the lymphatics.

3. Similar results were obtained during the absorption of water, of 0·9% NaCl solution and of serum from the lungs of rabbits. Whereas water was extremely rapidly absorbed, serum persisted in the lungs for about 4 days. This agrees with the slow absorption of oedema fluid from the lungs during the recovery phase of phosgene poisoning.

4. The introduction of a resistance to expiration of 4 or 6 cm. water pressure did not increase the absorption of serum introduced into the lungs of rabbits.

Our acknowledgements are due to the Director-General, Scientific Research and Development, Ministry of Supply, for permission to publish this investigation. We are also indebted to Prof. G. R. Cameron for the histological examination of the lungs.

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THE NUCLEOPROTEINS OF THE LIVER CELL DEMONSTRATED BY ULTRA-VIOLET MICROSCOPY

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It has previously been shown by chemical methods (Davidson & Waymouth, 1944*a*, *b*; Davidson, 1945) that liver tissue contains ribonucleic acid and that the ribonucleic acid content of the liver decreases on fasting while the content of nuclear deoxyribonucleic acid remains unchanged. The presence of ribonucleic acid in the cytoplasm of the liver cell has also been demonstrated by histochemical methods (Davidson & Waymouth, 1944*c*). As the constituent purines and pyrimidines of the nucleic acids absorb ultra-violet light strongly at a wave-length of 257 mμ. (cf. Caspersson, 1936), the ultra-violet microscope offers a convenient means of demonstrating the presence of these substances in the cell. Ultra-violet microscopy has been employed for the examination of cellular structure by a number of authors, e.g. Köhler (1904), Lucas & Stark (1931), Wyckoff, Ebeling & Ter Louw (1932), Wyckoff & Ebeling (1933), Lavin & Hoagland (1943) and Harvey & Lavin (1944), but as far as we are aware an investigation of the structure of the liver cell by this means has not hitherto been described.

METHODS

Young male rats weighing 125–150 g. were used. The animals were killed, the livers excised and small portions of tissue were fixed in formol-saline. In the experiments with regenerating liver, partial hepatectomy was carried out by the method of Higgins & Anderson (1931) under 'Nembutal' anaesthesia. The animals were killed 3 days later and the residual liver tissue excised and fixed.

The fixed tissue was embedded in paraffin in the usual way and sections 1 μ. thick were cut and mounted on quartz slides. The paraffin was removed and the sections soaked in glycerol. They were then covered with a quartz cover-slip and photographed by monochromatic ultra-violet light at a wave-length of 257 mμ. in the apparatus described by Barnard & Welch (1936).

Other sections from the same blocks were stained with haematoxylin-eosin or by Feulgen's method (de Tomasi, 1936) for ordinary histological examination.

RESULTS

The results are shown in Pl. 1. A section of liver tissue from a rat fed on a stock diet *ad libitum* is shown at A. The cell membrane stands out clearly. As might be expected, the nucleus contains a considerable amount of absorbing material, presumably deoxyribonucleoprotein (chromatin), and the nucleolus also absorbs intensely. In the cytoplasm, roughly spherical masses of strongly absorbing material are clearly visible. They are about 2 μ. in diameter and therefore correspond in size to the 'large particles' or 'secretory granules' of Claude (1943*a*, *b*).

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The effect of a 48 hr. fast is shown in B (Pl. 1). The cells have diminished considerably in size owing to a reduction in the amount of cytoplasm, and the sinusoids are wider. The diameter of the nuclei is unaltered and the nucleoli stand out as before. The large masses of absorbing material so clearly visible in A have almost completely disappeared from the cytoplasm.

If these masses in the cytoplasm of the liver cells from fed animals correspond to Claude's 'large particles', which are reported to contain ribonucleic acid, they might be expected to be affected by treatment with ribonuclease. This enzyme is known to remove from the cytoplasm of the liver cell granular material which stains intensely with toluidine blue (Davidson & Waymouth, 1944*c*). To investigate this matter further, paraffin sections of liver tissue from fed rats were mounted on quartz slides, taken down to water, and incubated for 5 hr. at 37° C. in isotonic veronal acetate buffer, pH 6·75, containing 1 mg. of crystalline ribonuclease (Kunitz, 1940) per ml. Control sections were incubated at the same time under the same conditions in buffer solution without enzyme. The sections were then mounted in glycerol and photographed. A control section is shown in C in Pl. 1. It presents the same appearance as in A and the cytoplasmic masses stand out clearly. On the other hand, in the section incubated in the enzyme solution (Pl. 1, D), these masses no longer absorb, although they can still be faintly distinguished. It should be noted that in enzyme-treated sections the nucleoli still contain absorbing material although the intensity of absorption is in general lower in the centre than at the periphery. In sections of liver tissue stained by the Feulgen technique (Pl. 1, F) the nucleoli also show the most intense reaction in the peripheral region.

A binucleate cell from a regenerating liver is shown in Pl. 1, E. The cytoplasmic masses and the nucleoli exhibit particularly strong absorption.

DISCUSSION

The effects of fasting on the histological appearance of the liver cell are of course well known, and the decrease in liver cytoplasm as the result of fasting has been described by, among others, Berg (1922, 1926, 1932, 1934), Noel (1923), Elman, Smith & Sachar (1943) and Kosterlitz (1944). Ultra-violet photography demonstrates, in addition, that fasting is accompanied by the disappearance from the cytoplasm of large granular masses which absorb ultra-violet light strongly at 257 mμ., indicating the possible presence of nucleic acids. Moreover, the size of the masses (2 μ. diam.) suggests that they are identical with the 'large particles' or 'secretory granules' of Claude (1940, 1943*a*, *b*), which are reported to be 0·5–2·0μ. in diameter and to contain protein, phospholipin and ribonucleic acid. (The relationship of these particles to the mitochondria is a matter of some dispute (Claude, 1943*a*, *b*; Bensley, 1942; Lazarow, 1943).) The disappearance of these masses on fasting can therefore be correlated with the observation that fasting is accompanied by a diminution in the liver's store of ribonucleic acid (Davidson & Waymouth, 1944*b*), of protein (Addis, Poo & Lew, 1936), and of phospholipin (Kosterlitz & Cramb, 1943). Berg (1922, 1926, 1932, 1934) described basophilic granules in the cytoplasm of the liver cell which disappeared on fasting. He considered that they consisted of protein, but it seems more probable that they were, in fact, phospholipin-ribonucleoprotein complexes (cf. Kosterlitz & Campbell, 1945).

The digestion of the cytoplasmic particles by crystalline ribonuclease is strong evidence that they contain ribonucleic acid, but the results of such a test must be interpreted with caution in view of the observation of Cohen (1945) that ribonuclease may act also on such material as thymonucleohistone. But since this substance is not a cytoplasmic constituent, since liver ribonucleic acid is known to act as a substrate for ribonuclease (Davidson & Waymouth, 1944*a*), and since ribonuclease removes from the cytoplasm basophilic material which stains deeply with toluidine blue (Davidson & Waymouth, 1944*c*), it seems justifiable to conclude that the absorbing material removed from the cytoplasmic masses by the enzyme is in fact the ribonucleic acid of the labile phospholipin-nucleoprotein complexes.

The 'microsomes' or 'small particles' of Claude (1943*a*, *b*) which, like the secretory granules, contain ribonucleic acid, are 50–200 mμ. in diameter and are therefore too near the limit of resolution with ultra-violet radiation of 257 mμ. to appear as well-defined structures. Moreover, particles of this size will have a strong scattering effect which is likely to mask any alteration in absorption due to the action of the enzyme. The cytoplasm of liver cells treated with ribonuclease shows little or no affinity for toluidine blue (Davidson & Waymouth, 1944*c*).

As the content of deoxyribonucleic acid (a purely nuclear constituent) in the liver is unaltered by a moderate fast (Davidson & Waymouth, 1944*b*; Davidson, 1945), a change in the intensity of absorption of the nuclear material would not therefore be expected and has not been detected in the cells examined.

The observations on the nucleolus are important, since much stress has recently been laid (cf. Caspersson & Santesson, 1942) on the ribonucleic acid content of the nucleolus in relation to protein synthesis. Using such material as sea-urchin eggs, the root-tip periblem cell of spinach, the cells of the *Drosophila melanogaster* salivary gland and tumour cells, it has been shown

(1) that the nucleolus gives a negative Feulgen test (Caspersson & Schultz, 1940), (2) that it absorbs ultra-violet light strongly at 260 mμ. (Caspersson & Schultz, 1940), (3) that the intensity of absorption is diminished on treatment with ribonuclease (Gersh, 1943), (4) that the basophily of the nucleolus is decreased after treatment with ribonuclease (Brachet, 1941), and (5) that the nucleolus gives positive histochemical tests for pentoses (Mitchell, 1942). This is fairly conclusive evidence for the presence of ribonucleic acid in the nucleolus of certain types of cell, but Koller (1943) has emphasized that nucleoli may contain either ribonucleic acid or deoxyribonucleic acid according to the type of cell. Hamberger & Hydén (1945) have reported that in the nerve cells of the cochlear ganglion of the guinea-pig, 19·1% of the nucleoli are Feulgen-negative and are composed of ribonucleoprotein, 64·2% are Feulgen-negative but are surrounded by a ring of Feulgen-positive chromatin, and 16·7% are Feulgen-positive. We have already shown (Davidson & Waymouth, 1944*c*) that the affinity of the nucleolus for toluidine blue is not reduced by treatment with ribonuclease, and it is clear from Pl. 1, D, that after such treatment the nucleolus still absorbs ultra-violet light strongly. This evidence that the nucleolus of the rat-liver cell appears to contain deoxyribonucleic acid rather than, or in addition to, ribonucleic acid is supported by the positive Feulgen test, although the results of the Feulgen reaction must be interpreted with certain reservations in view of the criticisms of Stedman & Stedman (1943, 1944) and of Carr (1945), which have, however, to some extent been answered by Stowell (1945). Since the Feulgen reaction is most clear at the periphery of the nucleolus, and since the central portion of the nucleolus absorbs ultra-violet light rather less intensely after treatment with ribonuclease, it may well be that small amounts of ribonucleic acid are to be found in the centre of the nucleolus. This would be in accordance with the views of Caspersson & Thorell (1941) and of Thorell (1944), who have shown that during the development of the embryonic cell and of the granulocyte the large Feulgen-negative nucleolus gradually shrinks and becomes surrounded by a ring of Feulgen-positive 'nucleolar associated chromatin' which constitutes what they term the 'chromocentre'. La Cour (1944) also observed that primitive cells in the blood series have Feulgen-negative nucleoli which are replaced on maturation by Feulgen-positive heterochromatic bodies. Similar observations on nerve cells have been reported by Hydén (1943) and by Hamberger & Hydén (1945).

SUMMARY

1. A description is given of the appearance in the ultra-violet microscope of sections of liver tissue from fed and fasting rats and from regenerating livers.

2. Fasting is accompanied by a decrease in the amount of cytoplasm and by a disappearance of cytoplasmic masses which are probably identical with the phospholipin-nucleoprotein complexes forming the 'large' or 'secretory'

granules. The nucleus is unchanged by fasting. These observations are in accordance with the chemical findings previously reported.

3. The presence of ribonucleic acid in the cytoplasmic masses is confirmed by their failure to absorb ultra-violet light after treatment with crystalline ribonuclease.

4. Feulgen-positive nucleoli and nucleoli which absorb ultra-violet light after treatment with ribonuclease have been found in rat-liver cells. Although deoxyribonucleoprotein appears to be the major constituent of the nucleoli, the probable presence of small amounts of ribonucleic acid is not excluded.

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EXPLANATION OF PLATE 1

A–E. Sections of rat-liver tissue, unstained, photographed by ultra-violet light at 257 mμ. × 1100.

A. Liver from an animal fed on stock diet. Masses of absorbing material about 2 μ. in diameter are seen in the cytoplasm. The nucleolus absorbs intensely.

B. Liver from an animal fasted for 48 hr. The volume of cytoplasm has shrunk and the sinusoids are wider. The absorbing material in the cytoplasm is reduced.

C. The same liver as in A. This section was incubated for 5 hr. at 37° C. in isotonic veronal acetate buffer pH 6·75. The appearance is similar to that in A. The central nucleolus contains four deeply absorbing nucleoli.

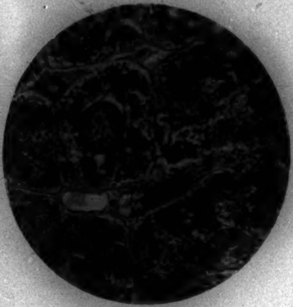
D. Section of the same liver as in C incubated for 5 hr. at 37° C., in a buffer solution containing 1 mg. crystalline ribonuclease per ml. The large cytoplasmic masses no longer absorb, but their outlines can be faintly distinguished. The nucleus contains two nucleoli. Both absorb less intensely than the nucleoli in C and the central portion of the upper one is almost free from absorbing material.

E. Large binucleate cell from regenerating liver. The absorbing material in the cytoplasm and the nucleoli are very prominent.

F. Section of liver tissue from the same animal as in A stained by the Feulgen technique and counterstained with orange G. The nucleoli stain more deeply at the periphery than at the centre. × 940.

THE JOURNAL OF PHYSIOLOGY, Vol. 105, No. 2

PLATE 1



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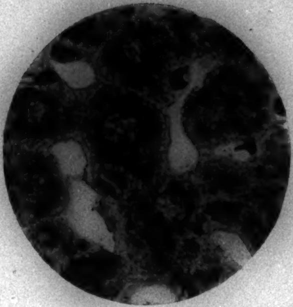

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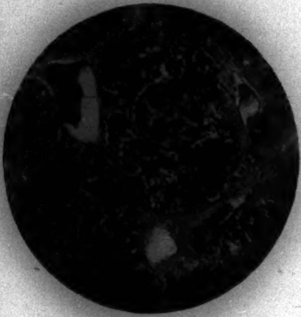

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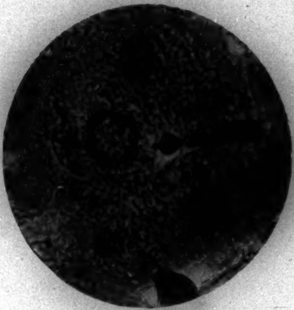

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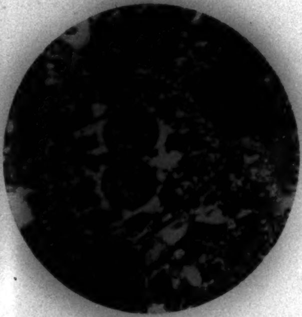

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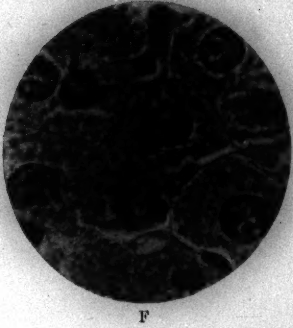

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To face p. 196

J. Physiol. (1946) 105, 197–204 615.782:546.29

PRELIMINARY OBSERVATIONS ON THE NARCOTIC EFFECT OF XENON WITH A REVIEW OF VALUES FOR SOLUBILITIES OF GASES IN WATER AND OILS

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(*Received* 3 *November* 1945)

The comparative narcotic effects of argon, nitrogen and helium have been previously studied by Behnke (1940) and by Behnke & Yarbrough (1938, 1939). Exposure up to 10 atm. of either air or an 80% argon, 20% oxygen mixture produces progressive narcotic effects in man. These effects are attributed to the dissolved inert gases, nitrogen and argon. Breathed in equivalent concentration, argon has approximately twice the narcotic effect of nitrogen and is twice as soluble as nitrogen in water and in fat. The fat-water solubility ratio of the two gases is nearly the same. Helium, on the other hand, with its lower solubility in water and fat has negligible narcotic effects when compared with argon and nitrogen.

The effect upon the central nervous system of argon, as compared with nitrogen, seems to depend primarily upon the total concentration of the dissolved gas. Reasoning from the Meyer-Overton hypothesis that anaesthetic effects are relative to the fat-water solubility ratio, it is suggested that gases such as krypton and xenon might have striking narcotic properties. These gases have a much greater water solubility and a higher fat-water solubility ratio. It is possible that 80% krypton at sea-level is as narcotic as 6 atm. of air, and that xenon under the same conditions may be equivalent to 25 atm. of air. Aside from the Meyer-Overton hypothesis, calculations of these theoretical effects are subject to considerable speculation in that the dissolved gas distribution ratio in the fat of the critical area in the central nervous system may be unlike that in olive oil. Fig. 1 shows a radio-autograph of a cross-section of a rat previously exposed to an atmosphere containing radiokrypton. After several hours' exposure the animal was killed, frozen and sectioned transversely and a section then placed in the cold, in close apposition to X-ray film. The relatively great uptake of the gas in fat containing tissue is noted by the dark areas in the radio-autograph.

A single subject in this laboratory, breathing 50% krypton and 50% oxygen in an experimental gas exchange measurement, voluntarily remarked that he had been quite 'dizzy' during the krypton inhalation. This is the report of a single subject. He had had, however, numerous experiences of breathing oxygen and nitrogen mixtures from the gasometric apparatus and, in this particular experiment, he was not aware of the fact that he was breathing some other mixture of gases.

Enough xenon was available to make a preliminary study of the effect of this inert gas upon mice. One of us (J.H.L.) exposed mice individually to a mixture of oxygen and xenon for short periods of time. The mixture was continuously circulated through soda lime, and the oxygen content frequently checked so that at least 20% oxygen was always present and CO_2 was removed. Additional oxygen was fed in as needed. The results are presented in Table 1. In the five mice exposed to an atmosphere with high xenon content, but with adequate oxygen, definite central nervous system effects were observed, and these findings might possibly be related to the high fat/water solubility ratio for xenon.

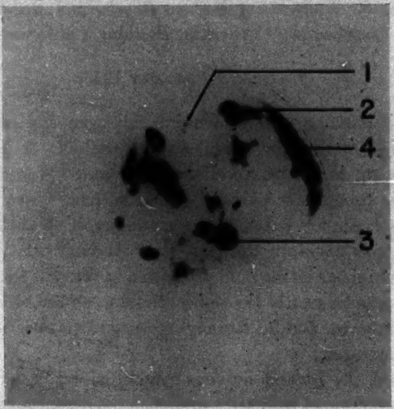

Fig. 1. The dark areas indicate clouding of the film due to radio krypton. Fat stains of section revealed those areas to have high fat content. (1) Spinal cord. (2) Perirenal fat. (3) Mesenteric fat. (4) Radiokrypton trapped in hair.

In connexion with certain studies on decompression sickness, it became necessary to know the solubilities of various inert gases in water and fats. By means of the use of radioactive isotopes, one of us (W.F.L.) determined the values for argon, krypton and xenon. An investigation of the literature and tabulation of the solubilities of various gases has given data which are of

TABLE 1. The effects on mice of breathing mixtures of oxygen and xenon, and of oxygen and krypton

No.	Gas mixture	Exposure	Narcotic effects and remarks
1	Approximately 50% krypton, 50% oxygen	1 hr.	None
2	Approximately 60% xenon, 40% oxygen	20 min.	Within 2 min. animal exhibited slight convulsive extensor movements of head. Developed weakness of hind legs. Complete recovery 15 min. after removal from chamber
3	At end of experiment 78% xenon, 22% oxygen	1 hr.	Within 2 min. mouse developed convulsive extensor movements of head; at 10 min. hind legs seemed partially paralysed; after removal from chamber had ataxia and unsteadiness with complete recovery in 15 min.
4	At end of experiment 69% xenon, 30% oxygen	1 hr.	Within 2 min. animal sluggish and convulsive. Motions of head as above. Later convulsive movements of hind legs developed
5	At end of experiment 60% xenon, 39% oxygen	45 min.	Within 2 min. developed convulsive movements of head and hind legs. On removal from chamber, ataxia for 3 min., then complete recovery
6	At end of experiment 58% xenon, 42% oxygen	30 min.	Within 1½ min. convulsive head movements as above. This continued throughout. After removal ataxia for 1 min., then apparent complete recovery

interest in the fields of aviation medicine and respiratory physiology. The results are presented in Figs. 2 and 3 and Table 2 and the bibliography is appended.

The values given are correct to two significant figures. Two or more references to a given value indicate that two or more investigators agreed (to two significant figures) to this value. Great discrepancies in the literature were found, especially in the values for neon and krypton. In cases such as these, the various figures were evaluated and those thought to be the most reliable were selected. The values for H_2, O_2, CO and CO_2 were taken from the *Handbook of Chemistry and Physics* (1944) and the *Dictionary of Chemical Solubilities* (1921). However, the *Handbook* values for the inert gases were in considerable error, having been taken from an earlier work of von Antropoff (1910). Antropoff's earlier values have been listed in the *Handbook of Chemistry and Physics* up to the 28th edition (1944), in the *Dictionary of Chemical Solubilities* (1921) and in the *International Critical Tables* (1928). Recent authors (Lannung, 1930; Valentiner, 1930; Van Liempt & Van Wijk, 1937), including von Antropoff himself (1919), have shown these values to be in error, especially those for neon and krypton. In the case of neon there seem to be only two recent sources of data—that of Lannung (1930) and that of Valentiner (1930). These two investigators differed considerably in their results, and it is difficult to decide which is the more reliable.

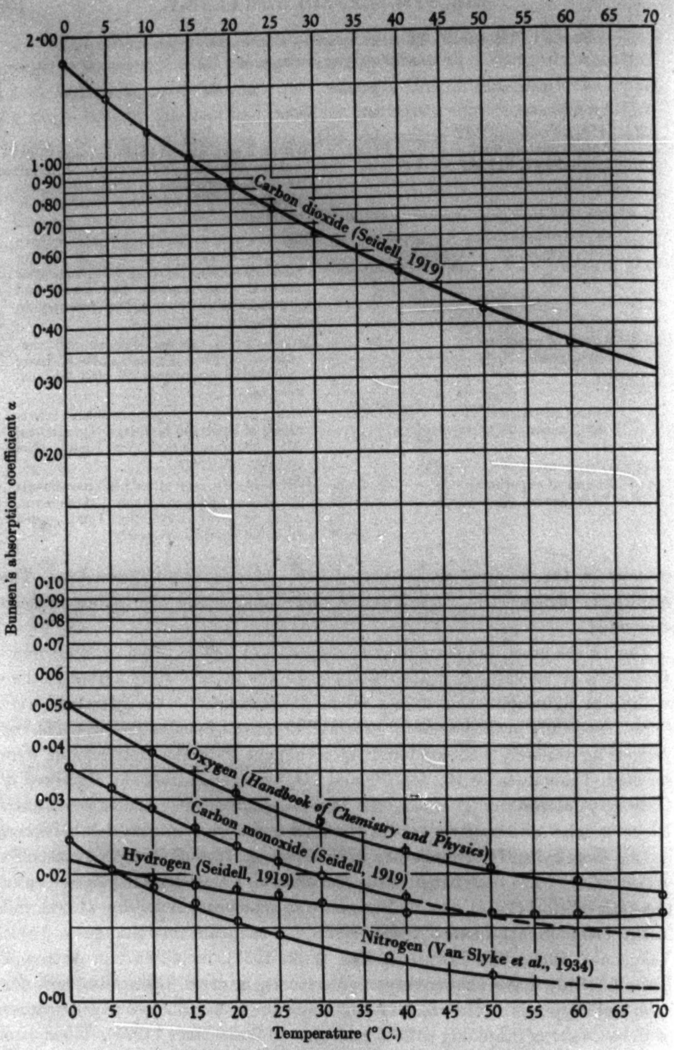

Fig. 2. Solubility of gases in water, α = c.c. gas (0° C., 760 mm.) dissolved in 1 c.c. water at temperature of observation.

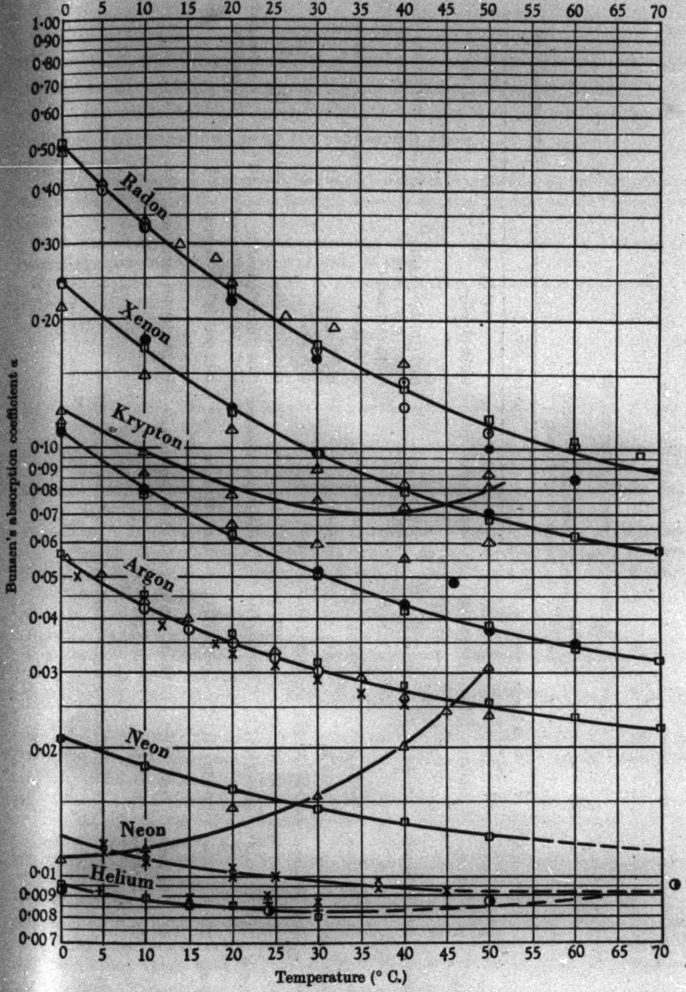

Fig. 3. Solubility of inert gases in water. ● von Antropoff (1919). △ *Dictionary of Chem. Sol.* (von Antropoff, 1910) (Argon, Comey). □ Valentiner (1927, 1930). × Lannung (1930). ○ Estreicher (1899). + Cady, Elsey & Berger (1922). ◐ Wiebe & Gaddy (1935). ⊙ Meyer (1913).

TABLE 2. Solubilities of gases in water and oils

	Gas	Mol. wt.	Half-life of isotope used	Bunsen's absorption coefficient (to 2 significant figures)				Oil/water solubility ratio	
				Solvent, water		Solvent, oil			
				22° C.	37° C.	22° C.	37° C.	22° C.	37° C.
Chemically inert gases	Helium	4	0·8 sec.	0·0086 (5, 1, 34)	0·0085 (13)	0·015 (3) olive	0·015 (3) olive		1·7 (4) olive
	Neon	20		0·0104 (15)	0·0097 (15)				
	Nitrogen	28	9·93 min.	0·015 (29)	0·013 (3)	0·052 (30) olive 0·063 (31) corn	0·07 (20) 32° paraffin 0·067 (3, 31) olive 0·063 (31) corn	3·5 olive 4·2 corn	5·2 (4) olive 4·7 (31) corn
	Argon	40	110 min.	0·037 (8)	0·026 (4, 15)	0·15* (20) olive	0·14* olive	4·0 olive	5·3 (4) olive
	Krypton	83·7	34 hr.	0·059 (28)	0·045 (33, 27)	0·44* olive	0·43* olive	7·5	9·6
	Xenon	131·3	34 days	0·13 (33, 27)	0·085 (33)	1·9* olive	1·7* olive	14·5	20·0
	Radon	222	3·825 days	0·23 (26, 19)	0·15 (26, 19)	0·25 (16) olive	0·19 (16) olive	110	125
	Hydrogen	2	—	0·018 (11)	0·016 (11)	0·042 (31) cottonseed	0·04 (20) 32° paraffin 0·05 (24) 40° cottonseed	2·3	3·1 cottonseed 2·5 paraffin
	Oxygen	32	—	0·030 (11)	0·024 (11)	0·11 (31) corn 0·11 (31) cottonseed	0·12 (24) 40° cottonseed	3·7	5·0
	CO_2	44	21·5 min.	0·83 (11)	0·56 (11)	1·34 (31) cottonseed	0·876 (24) 40° cottonseed	1·6	1·6
	CO	28	21·5 min.	0·022 (11)	0·018 (11)				

The numbers in brackets refer to the references on pp. 203–4.

* Determined in this laboratory by W. F. Loomis, using radioactive isotope.

SUMMARY

Observations on the narcotic effects of xenon are presented. The oil and water solubilities of hydrogen, oxygen, carbon dioxide, nitrogen, helium, neon, argon, krypton, xenon and radon are presented from a review of the literature and from determinations using the radioactive gases.

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We wish to thank our colleague, Dr H. B. Jones, for valuable discussions and assistance.

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THE METABOLISM OF KIDNEY SLICES FROM NEW-BORN AND MATURE ANIMALS

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In an earlier paper (Cutting & McCance, 1946), a study of the metabolism of kidney and liver slices from new-born and adult rats was described and the relevant literature discussed. The results were expressed per mg. of total N in the fresh slices, for it was shown that, during the incubation and shaking, a considerable part of the N initially present in the slices found its way into the medium surrounding them. The metabolism of new-born and mature rat kidney slices* differed in a number of ways. At maturity, the O_2 uptake began at about 3 cu.mm./min./mg. N but soon declined, and the slices lost from 40–50% of their N in 2 hr. At birth, the O_2 uptake was initially less rapid, but this lower rate was well maintained for 2 hr. and the loss of N was only some 18%. The addition of various substrates such as 0·02 M-Na-lactate, *dl*-methionine, or Na-succinate greatly raised the O_2 uptake of mature tissue, but had less effect on that from new-born rats. Posterior pituitary extracts diminished the loss of N and raised the O_2 consumption of mature tissues; large doses of desoxycorticosterone phosphate depressed the O_2 uptake. These additions made little difference to the new-born tissues.

To discover to what extent these findings applied to other mammals similar experiments have now been carried out on the cat, the pig and man. The experiments on these three kinds of animal will now be described, and the findings for the four will be compared and contrasted in the discussion. It has not been felt to be necessary to use large numbers of cats', pigs' and human kidneys as the experiments described in this paper were, on the whole, of a confirmatory nature. Consistent differences in behaviour between new-born and mature animals have been looked for in the first instance and it is hoped to investigate these at a later date.

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* For conciseness in the rest of this paper the kidney slices from any new-born animal will be referred to as new-born tissue and those from mature animals as mature tissue. Liver slices, where used, will be referred to specifically.

METHODS

The same technique has been used in all the experiments now to be described, and it is similar to that described by Cutting & McCance (1946). The medium in which all these experiments were carried out was Ringer's solution buffered with phosphate (Ringer phosphate). This was prepared as follows: To 103 vol. of 0·9% NaCl were added 4 vol. of 1·15% KCl, 1 vol. of 3·82% $MgSO_4$, $7H_2O$, 1 vol. of 2·11% KH_2PO_4 and 21 vol. of phosphate buffer pH 7·4, prepared by taking 40 c.c. 0·025 M-Na_2HPO_4 and 0·5 c.c. 2 N-HCl and diluting to 100 c.c. Ringer phosphate of pH 6·8 was prepared by substituting a similar volume of phosphate buffer pH 6·8. To the buffered solutions various substrates such as Na-lactate, Na-succinate, or *dl*-methionine were added as required.

RESULTS

Cats

Seven kittens from three litters have been used. All were less than 48 hr. old. They were killed by a blow on the head followed by severance of the carotid. All the results were similar, but litter mates resembled each other in behaviour slightly more than kittens from different litters. Three adult cats, all over 1 year old, were killed by a blow on the head or ether anaesthesia. The results were unaffected by the mode of death. The nature and results of the experiments on kidney slices are set out in Table 1 and some of the findings are shown in Fig. 1. It will be observed that the O_2 uptake of the new-born tissue fell off quite considerably during the first 15 min., but after this only very slowly during the remainder of the 2 hr. experiment. The O_2 uptake of the mature tissues fell off at about the same rate during the first 15 min. and continued to fall off throughout the experiment, so that the rate of O_2 uptake over the last hour was only one-third of that during the first 15 min. The O_2 uptake of the new-born tissues during the last hour was two-thirds of the initial uptake. More O_2 was taken up by the new-born than the adult tissue throughout the period of observation and the amount of N, which diffused out of the slices into the medium bathing them, was very much less.

TABLE 1. Respiration of kidney slices from new-born and mature cats

		Rate of O_2 uptake cu.mm./min./mg. N in the original slices					
Age of cat	Additions to the Ringer phosphate and pH	0–15 min.	15–30 min.	30–45 min.	45–60 min.	60–120 min.	% loss of N
Mature	None at 7·4	1·0	0·74	0·56	0·52	0·33	70·0
,,	0·02 M-Na-lactate at 7·4	2·17	1·78	1·76	1·39	1·14	45·5
,,	0·02 M-Na-succinate at 7·4	3·98	3·57	3·42	2·82	1·87	47·6
,,	0·9 unit 'Pitoxylin' at 6·8	1·68	1·45	1·45	1·45	1·35	24·0
New-born	None at 7·4	1·87	1·37	1·35	1·25	1·25	24·6
,,	0·02 M-Na-lactate at 7·4	2·33	1·85	1·69	1·84	1·66	23·4
,,	0·02 M-Na-succinate at 7·4	2·87	2·80	2·76	2·74	2·24	27·4
,,	0·9 unit 'Pitoxylin' at 6·8	2·03	1·55	1·55	1·64	1·54	14·1

Each figure in this table is the average of three experiments carried out on cats and of seven experiments carried out on kittens.

Na-lactate more than doubled the O_2 uptake of the mature tissue and decreased the N loss, but it did not have so much effect on the new-born tissues.

It raised their O_2 uptake to approximately the adult level, but this represents a very much smaller increase over the control. It had no effect on the N loss. Na-succinate nearly quadrupled the O_2 uptake of the mature tissues and it decreased the loss of nitrogen, but it did not prevent the O_2 uptake falling off. Na-succinate raised the O_2 uptake of the new-born tissues throughout the 2 hr. experiment—more so, in fact, than Na-lactate—but its effect was not nearly so great as it was on the mature tissues. It also slightly increased the percentage loss of N from new-born tissues, but it is doubtful if this is significant. 'Pitoxylin' (a posterior pituitary extract marketed by Messrs Oxo, Ltd) at pH 6·8 raised the O_2 uptake of mature tissue, prevented it falling off so rapidly and considerably decreased the N loss. Similar but much smaller effects were observed when the same amount of 'Pitoxylin' was added to new-born tissues. Thus the N lost by the mature tissue was reduced from 70·0 to 24·0%—but by the new-born tissue only from 24·6 to 14·1%. The effects of commercial 'Pitoxylin' are due to a number of causes which were analysed and discussed by Cutting & McCance (1946).

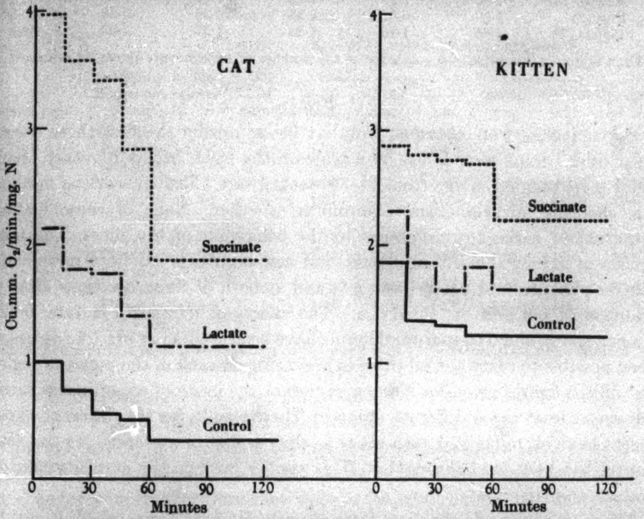

Fig. 1. The effect of lactate and succinate on the O_2 uptake of slices from the kidneys of new-born and mature cats

Table 2 summarizes the few experiments which have been carried out on liver tissue. The animals had all been fed a short while before they were killed. The new-born liver slices took up O_2 at all times more rapidly than the adult liver tissues, but at both ages the rate fell off as time went on. The mature liver slices lost less N than the mature kidney slices, but the kittens' livers lost approximately the same amount of N as the kittens' kidneys, and less than the amount lost by the mature livers.

TABLE 2. Respiration of liver slices in Ringer phosphate without added substrates at pH 7·4

Type and number of animals	Rate of O_2 uptake cu.mm./min./mg. N in the original slices					
	0–15 min.	15–30 min.	30–45 min.	45–60 min.	60–120 min.	% loss of N
Cats (3)	0·82	0·60	0·69	0·52	0·50	41·6
Kittens (5)	1·50	1·27	1·22	1·11	1·05	32·1
Pigs (2)	1·00	0·62	0·33	0·36	0·28	39·3
Piglets (2)	1·85	1·45	1·22	1·37	1·00	30·0

Each figure in this table is the average of the number of experiments shown in column 1

Pigs

Seven piglets were obtained from six litters during the first three days of their lives. Some were killed by a blow on the head, others by ether. Seven older pigs, ranging in age from 14–34 weeks, were killed by various methods, e.g. shooting, air embolism, chloroform or ether. None of these methods appeared to make any difference to the behaviour of the slices. The metabolism of kidney slices from mature and new-born pigs has been measured in Ringer phosphate at pH 7·4 and 6·8, and at both of these reactions after the addition of 0·9 unit of 'Pitoxylin'. The effects of 0·02 M-Na-lactate, 0·02 M-Na-succinate, and 0·02 M-*dl*-methionine have been studied at pH 7·4. It has not been possible to carry out all these experiments on each of the pigs and piglets, but all the figures given for kidneys represent the mean of experiments carried out on at least three different animals. The controls for the different experiments have all resembled each other so closely that one average control set of figures has been used throughout. This applies both to the new-born and the mature animals.

The metabolism of liver slices from recently fed new-born and adult pigs has been measured in Ringer phosphate at pH 7·4. The figures for pigs' liver closely resemble those obtained with cats' liver and both have, therefore, been included in Table 2.

Table 3 shows the results which have been obtained with kidney slices at pH 7·4 and 6·8 and the effects of 'Pitoxylin' at both these reactions. There are, therefore, a number of comparisons to be made. In all circumstances the new-born tissues had a higher O_2 uptake than those of the mature animal. At pH 6·8 the mature and the new-born tissues took up rather less O_2 at first and maintained this rate rather better than they did at 7·4. At both reactions the O_2 uptakes fell off but the rate was better maintained at birth. Thus at pH 6·8 the rate of O_2 uptake by the mature tissues in the second hour of the experiment was less than half of that taken up in the first 15 min., whereas the rate of O_2 uptake by the new-born tissues in the second hour was still more than two-thirds of that taken up initially. The effect of 'Pitoxylin' was to raise and partially to maintain the O_2 uptakes, but its initial effect was less at birth. The tissues from the new-born piglets lost less N than the mature tissues, but their losses were higher than those of other new-born animals. 'Pitoxylin' decreased slightly, though rather inconsistently, the losses of N from mature tissues at pH 7·4, but at 6·8 its effect was more characteristic; it also reduced the N losses of new-born tissues at both reactions.

TABLE 3. Respiration of kidney slices from new-born and mature pigs. Effect of pH and 'Pitoxylin'

			Rate of O_2 uptake cu.mm./min./mg. N in the original slices					
Age of pig	Additions to the Ringer phosphate and pH	No. of animals	0–15 min.	15–30 min.	30–45 min.	45–60 min.	60–120 min.	% loss of N
Mature	None at 7·4	7	1·14	0·76	0·61	0·44	0·43	54·2
,,	'Pitoxylin' at 7·4 (0·9 unit)	6	1·79	1·30	1·06	0·83	0·74	50·2
,,	None at 6·8	5	1·00	0·71	0·63	0·58	0·48	35·0
,,	'Pitoxylin' at 6·8 (0·9 unit)	5	1·61	1·29	1·18	0·95	0·70	26·6
New-born	None at 7·4	7	1·81	1·48	1·33	1·16	1·02	35·2
,,	'Pitoxylin' at 7·4 (0·9 unit)	4	2·04	1·80	1·65	1·62	1·44	24·6
,,	None at 6·8	5	1·59	1·39	1·34	1·25	1·12	27·3
,,	'Pitoxylin' at 6·8 (0·9 unit)	6	1·94	1·76	1·60	1·49	1·43	20·8

Each figure in this table is the average of the number of experiments shown in column 3

The effects of various substrates, Na-lactate, Na-succinate and *dl*-methionine are shown in Fig. 2. All increased considerably the amounts of O_2 taken up by the mature tissues, but Na-succinate had the greatest and Na-lactate the least effect. The same substrates also raised the O_2 uptakes of the new-born tissues. The changes were smaller than they were in the adult animal but the relationship between the different substrates was the same. Na-lactate did not lower the amount of N lost by the mature tissues but Na-succinate and *dl*-methionine reduced it from 54 to 42%. The influence of the substrates was as usual smaller on the new-born tissues. Na-lactate reduced the losses of N from 35 to 27% but the other substrates had no significant effect.

Man

Three human embryos aged 17, 24 and 30 weeks respectively have been investigated. Two were removed by hysterotomy and the third died at birth. In all cases the kidneys* were taken at once and measurements in the Barcroft apparatus were begun within 75 min. of death. Adult tissue was obtained from five kidneys which had been removed at operation, and from one kidney which was taken immediately after death. The five former had been removed for stones or tuberculosis, but there was sufficient normal tissue from which to cut slices, and the metabolism of these slices did not appear to differ from that of the slices from the kidney removed after death. This kidney, which appeared to be normal, came from a young man who had died of infective endocarditis. The respiration of all slices was being recorded within 1½ hr. of the removal of the kidney.

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* Slices from these kidneys will be referred to as foetal tissue.

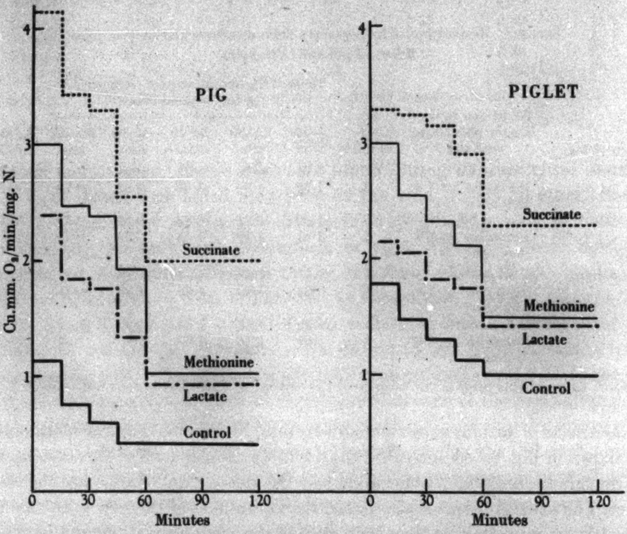

Fig. 2. The effect of lactate, succinate and methionine on the O_2 uptake of slices taken from the kidneys of new-born and mature pigs.

Experiments have been carried out with mature tissues in Ringer phosphate at pH 7·4 and 6·8, and the effects of 0·02 M-Na-lactate, 0·02 M-Na-succinate, 0·02 M-*dl*-methionine, 0·9 unit 'Pitoxylin', and 0·5 mg. and 0·05 mg. of desoxycorticosterone phosphate have been investigated at both reactions. Foetal tissue has been examined in Ringer phosphate at pH 7·4 and 6·8, and additions of 0·9 unit 'Pitoxylin' have been made at both reactions. The effects of Na-lactate and Na-succinate have been investigated at pH 7·4.

Table 4 gives the rate of O_2 uptake for the first 15 min., the following 45 min. and the final 60–120 min. The losses of N at the two ages are also shown. Each figure represents an average of at least two and usually of three or four experiments. The effect of pH has been more fully studied in man than in the other animals, particularly on the mature tissues. When the pH was lowered the O_2 uptake of the mature tissues was reduced during the first 15 min. (except in the presence of Na-lactate). Without exception, however, respiration was maintained better at the lower pH, so that at the end of 2 hr. the uptake at pH 6·8 was usually higher than it was at 7·4. Lowering the pH invariably curtailed the loss of nitrogen. Changing the pH had a slightly different effect on the foetal tissues, for the O_2 uptake of these slices tended to be a little higher at 6·8, though at this pH the loss of N was smaller. It will be seen that the O_2 uptakes of the tissues of adult man respiring without additions fell off throughout the 2 hr. but less than the O_2 uptakes of the corresponding tissues of the rat, pig or cat, and that they lost 42% of their N. The foetal tissues lost very much less N. Their O_2 uptake was about the same or perhaps slightly higher than that of the adults. It fell off most rapidly during the first 15 min. and in this resembled the O_2 uptake of the kittens.

TABLE 4. The effect of pH and of adding various substances upon the O_2 uptake of adult and foetal human kidneys

		pH 7·4					pH 6·8				
			Rate of O_2 uptake cu.mm./min./mg. N					Rate of O_2 uptake cu.mm./min./mg. N			
Age	Additions	No. of kidneys	0–15 min.	15–60 min.	60–120 min.	% loss of N	No. of kidneys	0–15 min.	15–60 min.	60–120 min.	% loss of N
Human adults	None	4	1·29	0·92	0·73	42·0	6	1·14	0·97	0·81	29·2
„	0·02 M-Na lactate	3	1·95	1·46	1·20	33·9	3	2·22	1·87	1·67	21·9
„	0·02 M-Na succinate	2	3·88	2·97	1·80	37·4	2	3·54	3·01	2·38	31·8
„	0·02 M-*dl*-methionine	2	1·70	1·01	0·73	37·4	3	1·31	0·83	0·67	32·6
„	0·9 unit 'Pitoxylin'	2	1·74	1·61	1·32	33·0	4	1·40	1·39	1·24	23·3
„	0·5 mg. desoxy-corticosterone-phosphate	1	1·05	0·77	0·45	39·2	2	0·92	0·72	0·65	29·1
Human foetus	None	3	1·52	0·92	0·83	19·3	2	1·66	0·75	0·81	17·2
„	0·02 M-Na-lactate	3	1·62	1·08	1·09	19·6	—	—	—	—	—
„	0·02 M-Na-succinate	3	1·92	1·48	1·38	20·5	—	—	—	—	—
„	0·9 unit 'Pitoxylin'	2	1·45	0·97	0·99	21·2	2	1·61	0·99	1·01	12·7

Each figure in this table is the average of the number of experiments shown in columns 2 and 7

Na-succinate and Na-lactate both raised the O_2 uptakes of the mature tissues. Na-lactate had a smaller initial effect but, as in rats and cats, a rather more lasting one. Both substrates reduced the loss of N but the effect of Na-succinate was small. Na-lactate and Na-succinate both raised, but only slightly, the O_2 uptakes of the foetal tissues. Na-succinate had the greater effect of the two. Neither substrate appreciably raised or lowered the loss of N at this age. *dl*-methionine raised the O_2 uptake of the mature tissues during the first 15 min. but after this had no further effect. 'Pitoxylin' at pH 7·4 and 6·8 raised the O_2 uptake of the mature tissues and decreased the loss of N, but it had no measurable effect on foetal tissue except to reduce the loss of N at pH 6·8. The effects of desoxycorticosterone phosphate on the adult human kidney were also investigated and were found to be similar to those on the kidney of the adult rat. 0·5 mg. at pH 7·4 and 6·8 reduced the O_2 uptake. 0·05 mg. had practically no effect at either pH and the results have not been given in the table.

DISCUSSION

The experiments which have already been carried out serve to show that there are certain ways in which the metabolism of kidney slices taken from newborn animals differs from that of slices taken from mature animals. It is evident, therefore, that ageing confers definite properties upon kidney cells which can be measured more or less accurately. It is equally clear, however, that each class of mammal responds to aging in a rather different fashion and some of these variations have been observed. The object of this discussion is to analyse these effects.

The O_2 uptake of the tissues respiring in Ringer phosphate solution at pH 7·4 was initially higher at maturity in the rat but at birth in the cat, pig and human. In the rat the rate of uptake was very much better maintained at birth than it was at maturity, and this was true also of the cat and man after the initial 15 min. had passed. In the pig the O_2 uptakes fell off at a similar rate at birth and maturity. In all four kinds of animal the O_2 uptake of the newborn tissue exceeded that of the mature tissues during the second hour of observation. These results are summarized in Fig. 3, and the insets show the percentage of the N, originally present in the slices, which was found in the fluid medium after 2 hr. It will be seen that in each species the loss of N was very much smaller at birth than at maturity. The percentage loss differed considerably from one species to another, and the relationships between the losses at the two ages differed to some extent from one class of mammal to another. The losses at birth most nearly approached the adult level in pigs, and it is suggested that this was due to the fact that piglets are at a much more advanced stage of development at birth than either rats or kittens, or than human embryos.

Lowering the pH from 7·4 to 6·8 had a very consistent effect upon the mature tissues. The change tended to depress the initial uptake of O_2 (although this was not observed in rats), to maintain it better and to reduce the loss of N. A similar change had inconsistent effects upon the O_2 uptakes at birth. In rats they were always slightly lower at pH 6·8 and in humans about the same at the two reactions. Only in piglets was the characteristic adult response observed, and this again may reflect the relatively greater maturity of their tissues. Lowering the pH did not materially alter the amount of N lost by the young rats or human embryos, but it reduced the piglets' losses.

The addition of various oxidizable compounds and hormone preparations brought out further consistent differences between the tissues at birth and at maturity. Na-lactate and Na-succinate always raised the O_2 uptakes of the mature tissues considerably more than those of the tissues at birth. The foetal material of man gave the smallest response to these substrates and piglet tissue the greatest, but it is quite certain that these compounds do not raise the O_2 uptakes at birth as they do at maturity, and the same appears to be true of *dl*-methionine, although the amino-acid has not been so extensively tested and

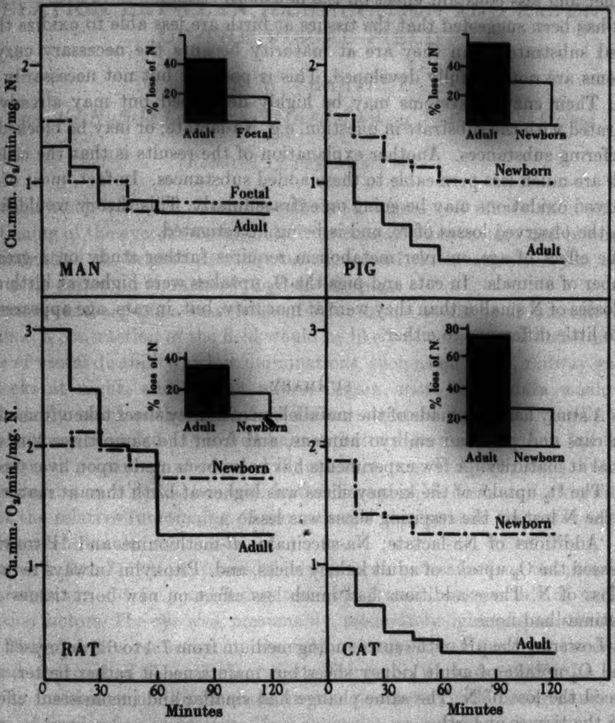

Fig. 3. The O_2 uptakes and the losses of N in Ringer phosphate at pH 7·4: Pigs, rats, cats and man.

appears to be oxidized only to a small extent by adult human kidneys. The effects of 'Pitoxylin' may be considered with the oxidizable substrates for its effects are probably due to a mixture of amino-acids and acetic acid (Cutting & McCance, 1946). It invariably raised the oxygen uptakes much more at maturity than at birth. It also materially reduced the losses of N at maturity except in the case of the pig at pH 7·4, and it tended to have a similar but smaller and less constant effect on the new-born tissues.

It has been suggested that the tissues at birth are less able to oxidize these added substrates than they are at maturity because the necessary enzyme systems are not yet fully developed. This is possibly but not necessarily the case. Their enzyme systems may be highly developed but may already be saturated with the substrate in question, e.g. Na-lactate, or may be blocked by interfering substances. Another explanation of the results is that the cells at birth are much less permeable to these added substances. In fact, most of the observed oxidations may be going on extracellularly. This theory would fit in with the observed losses of N, and is being investigated.

The effect of age on liver metabolism requires further study on a greater number of animals. In cats and pigs the O_2 uptakes were higher at birth and the losses of N smaller than they were at maturity, but, in rats, age appeared to make little difference to either.

SUMMARY

1. A study has been made of the metabolism of kidney slices taken from new-born cats and pigs and embryo humans, and from the same three kinds of animal at maturity. A few experiments have also been made upon liver tissue.

2. The O_2 uptake of the kidney slices was higher at birth than at maturity, and the N lost by the respiring slices was less.

3. Additions of Na-lactate, Na-succinate, *dl*-methionine and 'Pitoxylin' increased the O_2 uptake of adult kidney slices, and 'Pitoxylin' always reduced the loss of N. These additions had much less effect on new-born tissues and sometimes had none.

4. Lowering the pH of the surrounding medium from 7·4 to 6·8 depressed the initial O_2 uptake of adult kidney slices but maintained it rather better, and reduced the loss of N. The same change had smaller and inconsistent effects upon the tissues at birth.

5. Slices from kidneys of piglets differed less from those of pigs than the slices from kittens or human foetuses differed from those of cats or man.

6. Slices from the livers of new-born cats and pigs had a higher O_2 uptake and lost less N than the corresponding adult tissues.

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RELATION BETWEEN ILLUMINATION AND EXTENT OF THE VISUAL FIELD

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Although much is known about the effects of variation of illumination on the functioning of the eye, more particularly as regards visual acuity and intensity discrimination or brightness difference threshold, little attention has been paid to its action on the size of the field of vision.

From several aspects a knowledge of this relation would be of interest. For instance, a contraction of the field would be likely to constitute a factor in the cause of visual disability at low illuminations, such as on roads, railway yards or docks at night, and also in mines. Again, more exact data would be available by which pathological field defects might be detected clinically and their progress recorded. It is well known that a defect unnoticed in full light may become prominent at reduced illumination (e.g. Traquair, 1938), but few numerical data are available. And, further, it is possible that more information about the relative functioning of the rods and the cones in the outer retinal regions might result.

Ferree & Rand (1931) measured fields on a perimeter, keeping the illumination constant (at 7 f.c.) and using test-objects and backgrounds of various reflexion factors. The eye was, presumably, moderately light-adapted throughout. They found a reduction in the field as the reflexion factor of the background approached that of the target. The same workers (1939) also used various intensities of illumination on the perimeter with background and target of fixed reflexion factors. They showed a progressive reduction in field extent for a 1° blue stimulus under illuminations varying from 51 to 0·03 f.c. It is not quite clear, however, whether the fields depicted by them refer to the light or the colour threshold. Sloan (1939) measured the variations in light sensitivity across the retina using a 1° test-object against a background brightness of 0·7 m. lambert, but the result cannot readily be expressed in terms of the extent of the visual field. Weekers & Roussel-Liege (1945) have observed the effect of light- and dark-adaptation on the size of the field using a 2 m. Bjerrum screen at various illuminations but they were working with the eye in a non-stable state of adaptation.

For the present work it was decided to measure the field at various illuminations under conditions similar to those obtaining in everyday experience where objects and their background or surroundings are commonly illuminated by the same source of light and where, consequently, a variation of the illumination affects both object and background. The eye was adapted to the prevailing illumination.

METHODS

Apparatus. Use was made of a standard 330 mm. radius Lister Perimeter slightly modified as described below. As shown in Fig. 1 (*a*) the arc extended 90° on each side of the axis of rotation where the fixation point was fixed. The movable test-object could be made to travel from the fixation point outwards to one end of the arc while the source of illumination was situated near the opposite end. The eye of the subject was placed at the centre of curvature of the arc by means of an adjustable chin rest.

The chief alteration made was to substitute a 6 V. 3 W. lamp in a special fitting mounted inside the existing shield-reflector (Fig. 1 (*b*) and (*c*)) for the original 230 V. 40 W. lamp. The 6 V. lamp was completely enclosed and the only light escaping from it passed through a $\frac{3}{16}$ in. diameter aperture and fell on the surface of a small magnesium-oxide coated plate fixed inside an opal bulb at an angle of some 45° to the axis of the beam. Much of the light emitted through the opal bulb fell on the inner matt-white surface of the shield-reflector and suffered further diffusion before final reflexion on to the perimeter arc. A part of it reached the surface of the arc direct from the opal bulb. The design of the shield-reflector was such as to prevent any light from reaching the subject's eye directly.

TABLE 1

Position on working quadrant of arc in degrees from fixation point	Illumination in f.c., using 6 V. 3 W. lamp without filter
22°	0·0015
53°	0·0026
85°	0·0016

In the path of the light beam, between the 6 V. bulb and the magnesium-oxide surface, Wratten gelatine neutral filters could be inserted. The intensity of illumination on the perimeter arc could thus be reduced in steps without modifying the relative distribution of intensity from point to point over the arc, and without changing the spectral distribution of the light.

Although the source of light was near one end of the arc, the quadrant over which the test-object moved was reasonably uniformly illuminated. Actual photometric measurements were made at three positions by means of a special low range visual illumination meter (whose readings were checked at a somewhat higher level against another visual photometer of a different pattern). The results are given in Table 1 where each value shown is the mean of ten readings. The measurements were made without any filter in position, that is at the maximum illumination. The average illumination will be seen to be of the order of 0·002 f.c., the extreme values being 0·0015 f.c., and 0·0026 f.c.

The smallness of the spatial variation was, no doubt, explained largely by the shortness of the path from the light source to the centre of the arc being offset by the more oblique incidence of the light there. Since in all positions the surface of the movable test-object faced the centre of curvature of the arc the same effect applied to its illumination also. A further reason for the small variation was the relatively large size of the effective source of light which was mainly the inner white surface of the shield-reflector.

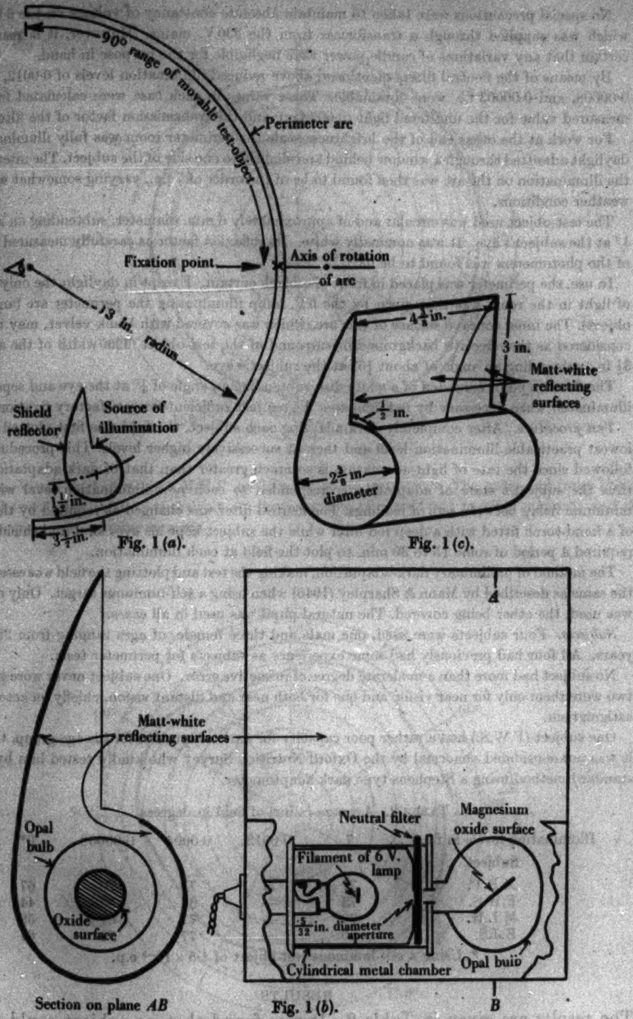
90° range of movable test-object
Perimeter arc
Eye
Fixation point
Axis of rotation of arc
13 in. radius
Shield reflector
Source of illumination
$1\frac{1}{2}$ in.
$3\frac{1}{4}$ in.
$4\frac{1}{8}$ in.
3 in.
Matt-white reflecting surfaces
$\frac{1}{2}$ in.
$2\frac{3}{8}$ in. diameter
A
Matt-white reflecting surfaces
Opal bulb
Oxide surface
Neutral filter
Magnesium oxide surface
Filament of 6 V. lamp
$\frac{5}{32}$ in. diameter aperture
Cylindrical metal chamber
Opal bulb
B

Fig. 1 (*a*).

Fig. 1 (*c*).

Section on plane *AB*

Fig. 1 (*b*).

Fig. 1 (*a*). Arc of perimeter showing position of source of illumination.
Fig. 1 (*b*). Shield-reflector with 6 V. lamp in special fitting.
Fig. 1 (*c*). Sketch view of shield-reflector.

No special precautions were taken to maintain absolute constancy of voltage on the 6 V. bulb which was supplied through a transformer from the 230 V. mains. However, it is reasonably certain that any variations of candle-power were negligible for the purpose in hand.

By means of the neutral filters mentioned above reduced illumination levels of 0·0012, 0·0004, 0·00008, and 0·00003 f.c. were obtainable. These values in each case were calculated from the measured value for the unfiltered light (0·002 f.c.), and the transmission factor of the filter.

For work at the other end of the brightness scale the perimeter room was fully illuminated by daylight admitted through a window behind and slightly to one side of the subject. The intensity of the illumination on the arc was then found to be of the order of 7 f.c., varying somewhat with the weather conditions.

The test-object used was circular and of approximately 6 mm. diameter, subtending an angle of 1° at the subject's eye. It was nominally white. Its reflexion factor as carefully measured by one of the photometers was found to be 63 %.

In use, the perimeter was placed in front of a black curtain. Except in daylight the only source of light in the room was that given by the 6 V. lamp illuminating the perimeter arc (and test-object). The inner concave surface of this arc, which was covered with black velvet, may thus be considered as the effective background or surround of the test-object. The width of the arc was 3½ in., subtending an angle of about 15° at the subject's eye.

The fixation point consisted of a white disk subtending an angle of ¼° at the eye and separately illuminated when necessary by red light to a degree just sufficient for satisfactory fixation.

Test procedure. After completely dark-adapting each subject, the field was first plotted at the lowest practicable illumination level and then at successively higher levels. This procedure was followed since the rate of light-adaptation is so much greater than that of dark-adaptation and thus the subject's state of adaptation corresponded to each new illumination level with the minimum delay between sets of readings. The neutral filter was changed as required by the light of a hand-torch fitted with a deep red filter while the subject kept his eyes shut and shielded. It required a period of some 15 to 30 min. to plot the field at each illumination.

The method of preliminary dark-adaptation, making the test and plotting the field was essentially the same as described by Mann & Sharpley (1946) when using a self-luminous target. Only one eye was used, the other being covered. The natural pupil was used in all cases.

Subjects. Four subjects were used, one male and three female, of ages ranging from 22 to 49 years. All four had previously had some experience as subjects for perimeter tests.

No subject had more than a moderate degree of refractive error. One subject never wore glasses, two wore them only for near vision and one for both near and distant vision, chiefly on account of astigmatism.

One subject (F.W.S.) had a rather poor capacity for dark-adaptation for his age group, though it was not considered abnormal by the Oxford Nutrition Survey who kindly tested him by their standard method using a Stephens type dark-adaptometer.

TABLE 2. Average radius of field in degrees

Illumination levels in f.c. ...	7	0·0012	0·0004	0·00008	0*
Subject					
M.R.P.	77	77	77	70	67
F.W.S.	73	—	67	53	44
M.L.H.	79	—	77	67	59
E.J.S.	80	—	77	71	61

* Using a self-luminous test-object of $1{\cdot}5 \times 10^{-9}$ c.p.

RESULTS

The results are given in Table 2. It was found that no subject could satisfactorily see the target at the lowest illumination available (0·00003 f.c.). At this low illumination the subject might catch sight of the test-object (with more

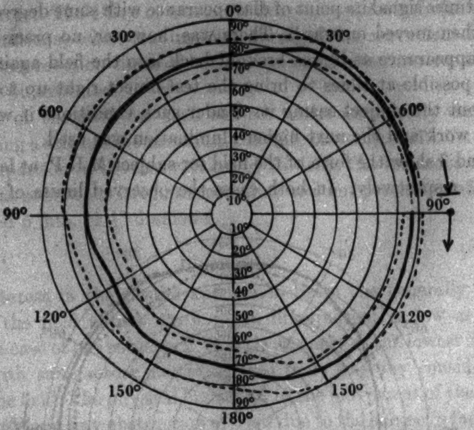

Fig. 2. Visual field of subject M.R.P. at 7 f.c., 1° white test-object. The outer and inner limits shown dotted relate respectively to disappearance of test-object moving outwards and its reappearance moving inwards.

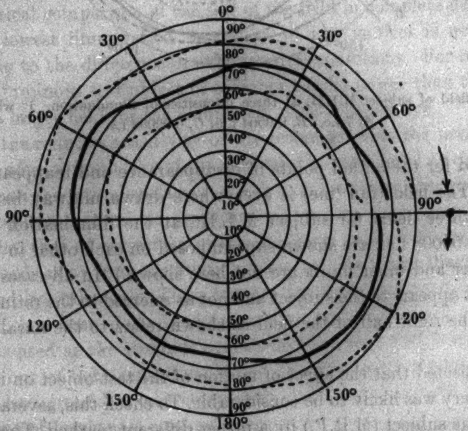

Fig. 3. Visual field of subject M.R.P. at 0·00008 f.c., 1° white test-object. The outer and inner limits shown dotted have the same significance as in Fig. 2.

or less difficulty) after it had been moved about well inside the field and he could sometimes signal its point of disappearance with some degree of certainty if it were then moved outwards. There was, however, no precision about its point of reappearance as it was brought back into the field again. Indeed, it was found possible at times to bring the test-object right up to the fixation point without the subject seeing it. Under such conditions it was useless to attempt to work and the next higher illumination was used.

Figs. 2 and 3 show the form of the field for subject M.R.P. at levels of 7 and 0·00008 f.c., respectively. In both cases the observed limits of the field are shown dotted for the actual points of disappearance and reappearance of the test-object. The field (full line) is everywhere drawn midway between these limits. The three fields for subject M.L.H., at the illumination levels of 7, 0·0004 and 0·00008 f.c., are shown superimposed on each other in Fig. 4. (The separate outer and inner limits are not here shown.) In all cases the field is depicted as it appears to the subject and not as referred to the retina. Thus, for a right eye the right half of the field as shown refers to the nasal side of the retina.

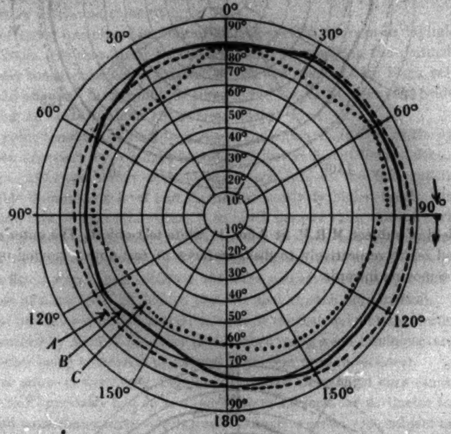

Fig. 4. Visual field of subject M.L.H. at three intensities of illumination. 1° white test-object *A*, 7 f.c.; *B*, 0·0004 f.c.; *C*, 0·00008 f.c.

It was suspected that the effect of motion of the test-object on its visibility at the periphery was likely to be considerable. To check this, several fields were plotted for one subject (M.R.P.) by a rather different method. The test-object was moved under cover along the arc to successive new positions and exposed only while stationary. This gave mean field radii of 77½° and 72° for the

7 f.c. and 0·00008 f.c. levels respectively, as compared with 77° and 70° for the moving target. The effect of motion is thus not appreciable (at either high or low illuminations) when readings are averaged for movement in opposite directions. Also, reaction times of both subject and experimenter are neutralized as would be expected.

The extent of the field for each subject under completely dark-adapted conditions using a self-luminous radium sulphide test-object of $1·5 \times 10^{-9}$ c.p. is given for comparison in the last column of Table 2. The luminous area of this test-object was somewhat irregular in outline and not precisely defined, but it did not exceed 3 mm. or ½° diameter.

DISCUSSION

The chief interest in the results appears to lie in the generally very small variation in the field extent down to an illumination as low as 0·0004 f.c. Except in the case of one subject (F.W.S.) it was only at the lowest illumination consistent with satisfactory recording that there was any considerable contraction of the field. The physical limitation of the movement of the test-object to 90° outwards and any obstruction of view due to the subject's facial features will both tend artificially to limit the observed extent of large fields more than of small ones. But even allowing for such matters the field at very low illuminations is larger than might have been expected.

It will be observed that the subjects showing the greatest contraction are those having the smallest fields under completely dark-adapted conditions. Direct numerical comparison of the size of the field in complete darkness with that at the lowest illumination in respect of any subject is not, however, implied owing to the difference in experimental conditions. For instance, the use of a self-luminous test-object of a higher candle-power than the one used would give a larger field for the completely dark-adapted eye.

Owing to the reduced sensitivity of the retina towards the periphery, each point on the edge or boundary of the field may be considered as corresponding to a position on the retinal surface at which a moving but otherwise unvarying stimulus produces a threshold sensation. (Actually there are two threshold positions; one, that of extinction of the sensation as the physical stimulus is moved outwards away from the fixation point, and the other, that of excitation as it is moved inwards again. These two are for the moment considered as one as, in effect, has been done in drawing the fields.) When a self-luminous test-object is used as the stimulus against a completely dark background this threshold is one of light sensation. When, however, as in the present experiments, the background is not completely dark the threshold may be considered as one of brightness contrast sensation.

At the low illumination of 0·00008 f.c., which is equivalent to a background brightness on the arc of the perimeter of 0·000004 effective f.c., or m. lambert (calculated on the assumption that the black velvet has a reflexion factor of 5%), vision will be mediated entirely by the retinal rods. On the other hand, at the relatively high illumination of 7 f.c., cone vision will be in operation, though it is not certain whether the rods also function at such a level. Thus the range of illumination used in the present experiments covers the so-called rod-cone transition level. The results indicate the desirability of acquiring further knowledge regarding both the point to point light threshold and the rod-cone transition brightness in respect of the outer peripheral regions of the retina.

SUMMARY

1. Four normal subjects were tested for the extent of the monocular field of vision at various illuminations ranging from 7 f.c., down to 0·00008 f.c., using a 1° test-object on a perimeter of 330 mm. radius where the test-object and its black background were both illuminated by the same source.

2. Only slight contraction of the field was observed for three subjects at an illumination level of 0·0004 f.c.

3. One subject who was known to have a poor but not abnormally poor capacity for dark-adaptation showed a greater contraction of the field as the illumination was reduced.

4. In general the contraction of the field at the lowest illumination level used (0·00008 f.c.) was related to the size of the subject's field when completely dark-adapted.

5. The changes of field extent are briefly discussed in relation to the rod-cone transition level in the periphery of the retina.

The author desires to acknowledge help received from several sources. Messrs British Thomson-Houston Co. Ltd., of Rugby loaned a low-reading photometer and, also, made the special opal bulb. Dr H. G. Kuhn of the Clarendon Laboratory, Oxford, helped with the design and construction of the low illumination fitting, while Dr W. D. Wright of the Imperial College of Science, London, loaned a photometer. Miss M. Barnett of this Laboratory has helped in preparing the diagrams. To all these the author tenders his thanks. Finally, he would like to thank Professor Ida Mann for laboratory facilities and valued suggestions.

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UREA EXCRETION BY RABBITS

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After some preliminary observations by Austin, Stillman & van Slyke (1921) on the excretion of urea by dogs and men, the 'urea clearance' was introduced as a clinical test of kidney function by Möller, Mackintosh & van Slyke in 1928. The latter found that, when U is the concentration of urea in the urine in mg./100 c.c., when B is the concentration of urea in the blood (which is assumed to remain constant during the observations) in mg./100 c.c., and when V is the volume of urine formed in c.c./min., their results could be expressed by the equation $UV/B=K$ (=75), when V was greater than 2·0 c.c./min. (=the 'augmentation limit') and by the equation $(U\sqrt{V})/B=K$ (=54) when V was less than 2·0 c.c./min. These equations, however, do not apply to rabbits. Although rabbits have often been used in experimental studies of kidney function, no equations for urea excretion in rabbits could be found in the literature. In the rabbit Richards & Plant (1915, 1922) demonstrated a relation between the rate of urine formation and blood pressure, MacKay & Cockrill (1930) showed that the rate of excretion of urea was related to its plasma concentration, and Kaplan & Smith (1935) found that urea 'clearances' (UV/B) were diminished when the volume of urine was small. But, from the point of view of using the rate of urea excretion as a test of kidney function, there were insufficient data to enable physiological variations to be distinguished from changes which might be pathological, e.g. after poisoning by a toxic drug.

The difficulty is best expressed by an example. In a rabbit (M) weighing 2 kg. six observations were made of U and V, and two of B. In this example, which is purposely chosen as extreme, U varied from 4160 mg./100 c.c. to 317 mg./100 c.c., and V from 0·74 to 0·025 c.c./min. Since these were consecutive observations, an equation which truly expressed the functional capacity of the kidneys should give the same answer in all of them. The following are the 'clearances' employing the equation $UV/B=$clearance:

1·57 1·76 2·79 4·4 3·67 2·91

The 'clearances' do not even approximate to a constant, the biggest being 2·8 times the smallest. Nor is the situation improved if the equation $(U\sqrt{V})/B$ = clearance is adopted. The results then become:

8·88 11·15 6·46 5·10 4·34 4·37

where, again, the largest is 2·57 times the smallest. Therefore it was clear that until some equation was found to express the physiological relationship between U, V and B, the rate of urea excretion could not be used as a kidney function test.

METHODS

Observations of U, B and V were made on eleven male rabbits of average weight 1·9 kg. They were given greens and water *ad lib.* for several days before the observations were made in order to avoid the dehydration common in laboratory animals. The rabbit selected for experiment was deprived of food, but not of water, for 24 hr. beforehand. On the morning of the experiment it was given 40 c.c. warm tap water/kg. by stomach tube and placed in a metabolism cage designed to collect urine free from faeces. Ninety minutes later it was given a further 40 c.c. water /kg., and, after another 30 min., the first blood sample was taken from an ear vein. The bladder was then emptied, a careful note being made of the time, and the urine discarded. At intervals of about 30 min. thereafter the bladder was emptied, time and volume being noted, and the urine kept for estimation of urea. When four or five urine samples had been obtained, a second blood sample was taken; this bleeding was purposely done at the beginning and end of the urine collection instead of in the middle, as manipulations during bleeding were inclined to inhibit the diuresis. The appropriate values of B were then obtained by interpolation on a graph.

For the observations recorded in this paper the bladder was emptied by catheterization (no. 1 gum elastic, kept in hot water, and dipped in liquid paraffin) and by supra-pubic pressure. It is, perhaps, worth mentioning here that a better method, unhappily not hit upon until this series of observations was finished, is as follows. A no. 2 or 3 rubber catheter is passed, while an assistant holds the rabbit supine on the bench, and the free end of the catheter is then attached, via a collecting flask and a water-trap, to a suction pump. By manœuvring the catheter round and back and forth it is possible to ensure that the last drops of urine are sucked out.

Urea in blood and in urine was estimated by the Conway (1942) and van Slyke methods. For the latter some specially designed deep aeration tubes, with ground-glass stoppers, were employed.

RESULTS

Urea excretion in rabbits. Altogether ninety-eight observations of U, B and V were obtained, and have been recorded in Fig. 1. It was noted that the values of V fell from 1·0 down to 0·025 c.c./min. This range has been split up into a series of steps of 0·05 c.c./min. and at each step the mean of all the values of the ratio U/B which lie within 0·05 c.c./min. on either side of it have been plotted as circles. Thus each circle in the graph represents the mean of all those values of U/B which were observed within 0·05 c.c./min. of the value of V at which the circle is plotted. The course on which the circles lie suggests a regression line of the form

$$\log V + c \log U/B = \log K.$$

The crosses in Fig. 1 represent the same points as the circles, but have been plotted as logarithms. They lie reasonably close to a straight line, which has been drawn by eye and represents the equation $\log V + 1{\cdot}4 \log U/B = 8{\cdot}4$. The continuous curve drawn in Fig. 1 passes through values of U/B calculated for each value of V from the equation $(U/B)^{1{\cdot}4} \times V = 6{\cdot}92$. This equation represents the series as a whole, but it is, of course, possible to work out the constants c and K for each rabbit individually. Unfortunately, very little control can be exercised over the rate of urine formation in a rabbit and the constant c cannot be determined with any accuracy, unless the observations cover a fairly wide range of V. In Table 1 are shown some representative series, starting with the observations on rabbit M, which have already been mentioned as an example of the inapplicability of the equations $UV/B = K$ and $(U\sqrt{V})/B = K$. The first column gives observations of U, and the second the interpolated values of B; instead of values of V, the actual volumes and times in minutes between urine collections have been recorded in the next two columns. The constant c has been calculated for each rabbit, and the appropriate value of K for each set of observations is given in the final column. From these results it appears that in rabbits a urea excretion 'constant' can be found which is equivalent to an estimation of the 'clearance' in man. When the appropriate value of c has been determined, the equation $(U/B)^c \times V = K$ can be expected to give results at least more consistent than the clearance equations $UV/B = K$ and $(U\sqrt{V})/B = K$, which are, in fact, special examples of the same basic equation, in which the values of c happen to be 1·0 and 2·0 respectively.

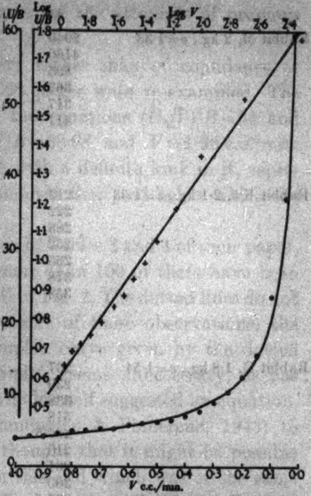

Fig. 1. Rabbits. Ordinates: Values of the ratio U/B. Abscissae: Rate of urine formation in c.c./min. (V). The range of values of V has been divided into 'steps' of 0·05 c.c./min. Each circle represents the mean of the observations of U/B which lie within 0·05 c.c./min. on each side of the value of V at which the circle is plotted. The crosses represent exactly the same figures as the circles, but are plotted as logarithms. The continuous curve is drawn from the equation $(U/B)^{1{\cdot}4} \times V = 6{\cdot}92$.

Urea excretion in man. These results from rabbits led naturally to the conjecture that some similar equation might apply to urea excretion in man. There were, however, good reasons for diffidence in approaching the subject. In their 1928 paper, Möller, Mackintosh & van Slyke are unequivocal. They say: 'The results of our experiments...confirm the conclusions of Austin, Stillman & van Slyke. There is a distinct augmentation limit. Below it, clearance increases

TABLE 1. Observations on individual rabbits

	U	B	Actual volumes of urine (c.c.)	Time in min. between urine collections	K
Rabbit M, 2 kg., $c=1\cdot33$	2940	58·2	1·0	32	5·78
	4160	58·9	0·75	30	7·17
	895	60·0	15·0	80	6·83
	363	61·2	37	50	7·90
	317	62·0	16·5	23	6·30
	411	62·4	18·5	42	5·42
					Mean = 6·57
					$\sigma=13\cdot4\%$
Rabbit K5, 2·1 kg., $c=1\cdot53$	219	41	13	30	5·64
	222	40·3	14	30	6·32
	288	39·6	8	30	5·57
	252	38·7	28	60	8·25
	265	38·2	14	40	6·80
	318	37·7	9·5	40	6·24
	358	37·3	3·7	20	6·64
					Mean = 6·49
					$\sigma=13\cdot9\%$
Rabbit G, 1·8 kg., $c=1\cdot54$	237	27	29	57	14·40
	230	26·6	9·5	20	13·11
	338	26·5	4·4	25	8·91
	518	26·2	13·0	95	13·60
	265·5	26·0	21·5	55	13·90
	210	26·0	16·0	30	13·38
	261	26·0	12·0	34	12·24
	360	26·0	9·0	42	12·25
	997·5	26·0	2·1	40	14·56
	546	26·0	8·6	42	22·15
					Mean = 13·85
					$\sigma=24\%$
Rabbit F, 1·9 kg., $c=2\cdot1$	225	31·3	15	30	31·6
	255	33·2	8·5	24	25·4
	330	37·0	32	89	35·5
	187	32	21	25	34·6
	217	32	11	20	30·5
	265	32	9	25	30·5
	165	32	49	75	20·55
	412	34	4	67	31·40
	456	36·2	3·1	45	30·00
					Mean = 30·0
					$\sigma=15\cdot2\%$
Rabbit H, 2·0 kg., $c=1\cdot91$	180	31	5·1	24	6·1
	400	29·2	3·0	76	5·83
	81	28·7	15	20	5·42
	85·5	29·4	15	23	5·00
	111	29·8	7·5	20	4·63
	135	30·5	10	32	5·34
	120	31·6	35	65	6·90
					Mean = 5·6
					$\sigma=13\cdot6\%$

with increasing urine volume, while above it the clearance is independent of urine volume.' And, referring to their expressions $(U\sqrt{V})/B$ and UV/B they add: 'No other line or curve could follow the experimental points more closely.' Moreover, in their discussion of the literature they include a list, reminiscent of skeletons beside a desert track, of the attempts of previous workers to find a 'Urea excretion constant'.

It was, therefore, in a spirit of curiosity rather than of confidence or criticism, that the results given in the 1928 paper were re-examined. The dotted lines given in Fig. 2 are drawn to the equations $(U\sqrt{V})/B=54$ and $UV/B=75$. These lines intersect when $U/B=38{\cdot}88$ and $V=1{\cdot}96$ c.c./min. Together they constitute a composite curve with a definite kink in it, representing the 'augmentation limit', above which the first, and below which the second, equation applies.

From the lists of values of U, B and V given in Tables 3 and 4 of their paper, the ratios U/B have been calculated, and more than 100 of them have been plotted against the corresponding values of V in Fig. 2. The dotted lines do not lie very close to a pathway through the centre of these observations, the 'spread' of which is so great that the complex curve given by the dotted lines, with a kink at the 'augmentation limit', seems unnecessary, as has already been shown by Dominguez (1935), who himself suggested an equation, which was modified in a later paper (Dominguez & Pomerene, 1943) to $UV/B=A\ (1-e^{-kv})+bv$. However, it was thought that it might be possible to find an equation which was simpler than this and which was of the same form as that already described for rabbits. The equation $(U/B)^{1{\cdot}103}\times V=81{\cdot}3$ has been drawn as a continuous line in Fig. 2. It is not claimed that this is the only, or even the best, possible equation, but it has several features to recommend it. It is a single simple equation, applicable to all values of V, and it disposes of the 'augmentation limit' and of the necessity for two methods of calculation. Its course does pass approximately through the middle of the observed points. At the 'standard' rate of urine flow, namely 1·0 c.c./min., it gives the same value to the ratio U/B as does the old equation $(U\sqrt{V})/B=54$, namely, 54. Finally, it does represent the observations more closely than either the equation $UV/B=75$ or $(U\sqrt{V})/B=54$. Each observation of U/B above the critical value 38·88 (the augmentation limit) was used to calculate V from the equation $(U\sqrt{V})/B=54$; this calculated value of V was then compared with the observed value of V, in order to obtain a figure (D) representing the difference between observation and calculation; e.g. when $U/B=95{\cdot}9$, according to the equation $(U\sqrt{V})/B=54$, the value for V should be 0·316. It was, in fact, 0·48; thus the value of D was 0·164. Similarly, for all observations of U/B *below* 38·88, V was calculated from the equation $UV/B=75$, and a figure D obtained in each instance by comparison with the observed value of V. Finally, V was calculated for *all* values of U/B from the equation

$(U/B)^{1\cdot 103} \times V = 81\cdot 3$, and once more figures for D obtained. Every single set of observations given in the 1928 paper was calculated in this way. The results are given in the table on p. 229. Thus it is clear that the equation

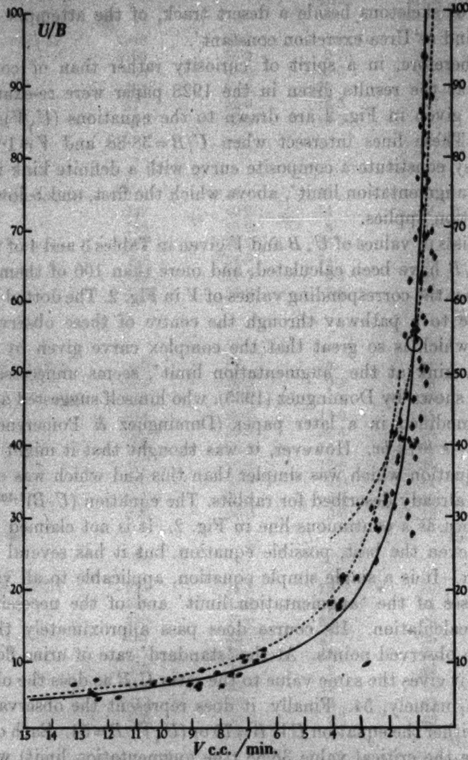

Fig. 2. Man. Ordinates: Values of the ratio U/B. Abscissae: Rate of urine formation in c.c./min. (V). The upper dotted line is drawn from the equation $(U\sqrt{V})/B = 54$. The lower dotted line is drawn from the equation $UV/B = 75$. The points are plotted from tables 3 and 4 of the paper quoted in the text. The continuous curve is drawn from the equation $(U/B)^{1\cdot 103} \times V = 81\cdot 3$.

$(U/B)^{1\cdot 103} \times V = 81\cdot 3$ represents the observed relationship of U, B and V more closely than the two previous equations, to the extent of 24% when U/B is greater than 38·88, and of 15% when it is less.

Mean values of D.

	Using the equation $(U\sqrt{V})/B=54$	Using the equation $(U/B)^{1\cdot 103}\times V=81\cdot 3$
When U/B is between 38·88 and 100	The mean value of D is 0·265	The mean value of D is 0·214
	Using the equation $UV/B=75$	Using the equation $(U/B)^{1\cdot 103}\times V=81\cdot 3$
When U/B is between 0·0 and 38·88	The mean value of D is 1·157	The mean value of D is 1·012

CONCLUSIONS

It appears that in man, as in the rabbit, the relationship between U, B and V can be expressed by an equation of the form $V(U/B)^c=K$. It is not suggested that the equation $(U/B)^{1\cdot 103}\times V=81\cdot 3$ is the only possible equation for man, but it does correspond to the observations of U, V and B given by Möller, Mackintosh & van Slyke more closely than the equations $(U\sqrt{V})/B=54$ and $UV/B=75$. If it does represent the behaviour of the kidneys in excreting urea, it is apparent that the increase in the concentration U is a function of the reabsorption of water over the whole range of values of V. From clearances of inulin and other substances believed to be filtered by the glomeruli, but not reabsorbed, the rate of glomerular filtration is known to be about 120 c.c./min. Thus, if no water were reabsorbed at all, V would become 120 c.c./min. Substituting this value in the equation $(U/B)^{1\cdot 103}\times 120=81\cdot 3$, then $U=0\cdot 705\,B$.

Assuming that such a large extrapolation is justifiable, then more than a quarter of the urea filtered at the glomeruli is removed from the tubular fluid even in conditions in which no water is reabsorbed, i.e. in which the concentration of urea in the tubules does not become greater than that in the blood.

SUMMARY

1. The equations used to express urea clearance in man cannot be applied to the rabbit.

2. Experimental details are described of the methods by which a series of observations of U, B and V was made on rabbits.

3. It is shown that, in the means of observations on eleven rabbits, the relationship between U, B and V can be expressed by the equation

$$(U/B)^{1\cdot 4}\times V=6\cdot 92.$$

Representative lists of observations on individual rabbits are also recorded.

4. Observations of U, B and V in man made by Möller, Mackintosh & van Slyke (1928) are discussed, and shown to correspond more closely to the equation $(U/B)^{1\cdot 103}\times V=81\cdot 3$ than to the equations $(U\sqrt{V})/B=54$ and $UV/B=75$. If this equation be assumed to hold over all possible values of V, it implies that more than a quarter of the urea filtered by the glomeruli is removed from the tubular fluid even in the absence of reabsorption of water.

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THE EFFECT OF ADRENO-CORTICOTROPHIC HORMONE ON THE RESPIRATORY METABOLISM OF THE ADRENAL CORTEX OF THE RAT

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The present investigation arose out of experiments on the action of the anterior pituitary corticotrophic principle on the adrenal cortex. It seemed of particular interest to ascertain the latent period of this effect for there is some evidence that it might be quite short. For example, the cholesterol content of the adrenal cortex has been found to be considerably reduced 3 hr. after injection of corticotrophic hormone (Sayers, Sayers, White & Long, 1943; Carreyett, Golla & Reiss, 1945). Moreover, the severity of artificially induced shock-like symptoms in rats was greatly reduced by pre-treatment with corticotrophin half an hour before the experiment (Reiss, Macleod & Golla, 1943). Measurement of oxygen uptake—the most obvious criterion of energy exchange and cell activity—has therefore been used to investigate this point, and the aerobic lactic acid production was studied at the same time.

METHODS

The rats used were male Wistar rats weighing approximately 50 g.

The adreno-corticotrophic hormone preparations used were prepared according to Carreyett *et al.* (1945) and Reiss & Golla (1945). New preparations were compared with a laboratory standard which was stored over P_2O_5 *in vacuo*.

The biological assay method used for testing the different corticotrophic preparations was in accordance with the method originally described by Reiss, Balint, Östreicher & Aronson (1936), based on the disappearance of the sudanophobic zone in the adrenals of hypophysectomized rats. The original biological unit similar to that later used by Simpson, Evans & Li (1943) did not appear sufficiently defined, being only a threshold dose and not taking account of the changing sensitivity of the test animals. Dose response curves were therefore drawn for every consecutive preparation, by plotting the percentage of positive responses against the logarithm of the dose, and compared with the dose response curve for the laboratory standard preparation. 2 mg. of the latter was taken as the unit and after comparison of the 50% positive response of both preparations the unknown preparation was sufficiently determined. An example of the standardization described is given in Fig. 1.

The oxygen consumption was measured by the direct method of Warburg, in glucose phosphate Ringer's solution at pH 7·4. Conical flasks (10 ml.) were used, containing 2 ml. Ringer phosphate, with 0·2 ml. 20% potassium hydroxide in the small inner tube. The gas phase consisted of oxygen. The lactic acid produced aerobically was estimated in the Ringer's solution at the end of the experiment by the colorimetric method of Barker & Summerson (1941).

Since tissue slices could not be cut, the adrenals were bisected so that the two halves remained connected by a small strand of cortical tissue. The medulla could be then scooped out from the two halves by blunt dissection, leaving a thin layer of adrenal cortex the thickness of which did not exceed the maximum permitted for free diffusion. It is possible that during removal of the medulla, part of the zona reticularis of the cortex was removed, so that the metabolism measured was mainly that of zona glomerulosa and zona fasciculata. The cortical tissue prepared from six adrenals was found to be a convenient amount to use in flasks of the stated capacity. The dry weight of adrenal cortical tissue was found to be about 3–4 mg. per flask. The nitrogen content of the total adrenals was determined by micro-Kjeldahl combustion.

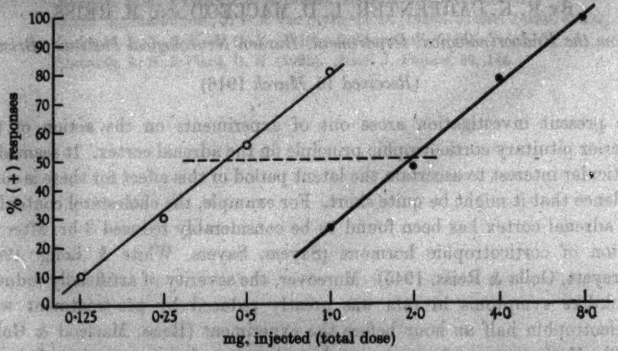

Fig. 1. Comparison between corticotrophic preparation of unknown strength with laboratory standard preparation. —●— standard preparation. —○— corticotrophic preparation of unknown strength. 50% response with 2 mg. standard (1 unit) and 0·4 mg. unknown preparation.

RESULTS

It will be seen from Table 1 that the results obtained in normal rats are reasonably consistent, the mean Q_{O_2} observed being 3·46 ± 0·117 (s.e. of ten

Table 1. Oxygen uptake and lactic acid production of adrenal cortical tissue

Normal rats (40–50 g.)				Rats treated with 100 units corticotrophic hormone for 24 hr.			
No. of animals	Av. wt. of pooled adrenals (mg.)	Cortex-Q_{O_2}	Cortex μg. lactic acid/mg. dry wt./hr.	No. of animals	Av. wt. of pooled adrenals (mg.)	Cortex-Q_{O_2}	Cortex μg. lactic acid/mg. dry wt./hr.
6	7·3	3·35	2·6	3	7·7	3·93	3·95
5	7·0	4·18	3·2	5	7·9	4·44	4·36
5	6·9	3·14	2·9	5	7·9	4·44	2·38
3	6·8	3·50	4·7	2	9·8	4·99	7·28
3	6·3	3·67	4·4	2	8·5	4·45	9·67
3	7·7	3·76	6·3	3	8·7	3·65	6·10
3	7·7	3·35	4·6				
3	7·5	3·36	4·9				
3	6·8	2·82	6·6				
3	7·1	3·52	4·4				

Mean $Q_{O_2} = -3·46$; $\sigma = 0·37$; $\epsilon = 0·117$.

Mean $Q_{O_2} = -4·32$; $\sigma = 0·468$; $\epsilon = 0·191$.

$n = 14$; $t = 3·84$; $p < 0·01$.

experiments). Twenty-four hours after treatment with 100 units of corticotrophic hormone a mean value for the Q_{O_2} of $4{\cdot}32 \pm 0{\cdot}191$ (6) was observed. This difference of about 25 % between the means proved to be significant when Fisher's *t* test (1941) was applied.

There was no obvious relation between the dose of corticotrophin injected and the magnitude of the oxygen consumption observed. It will be seen from Table 2 that the oxygen consumption in some experiments was raised to the maximal value observed 6 hr. after injection of the hormone. There was some indication that after the initial response to a high dose of corticotrophic hormone the Q_{O_2} later fell to normal values.

TABLE 2. Oxygen consumption and glycolysis of adrenal cortex after the injection of corticotrophic hormone

No. of animals	Av. adrenal wt. (mg.)	Units corticotrophic hormone injected	Duration of treatment (hr.)	Q_{O_2}	µg. lactic acid/mg. dry wt./hr.
5	7·4	50	6	3·46	1·98
5	8·9	50	24	5·47	7·14
2	8·7	50	24	3·52	6·45
3	7·3	100	6	7·72	4·76
3	10·5	100	6	3·72	5·60
3	8·5	100	6	4·45	10·60
3	7·7	100	48	4·70	10·70
5	8·0	100	72	3·31	6·71
3	10·3	200	24	5·90	9·90
3	10·3	200	72	5·40	7·16
6	8·3	200	96	3·44	5·45
5	8·4	150	72	3·84	2·57

The aerobic glycolysis did not show such a definite response as the oxygen uptake, but it is noteworthy that high figures for aerobic glycolysis were found only in animals injected with corticotrophic hormone.

TABLE 3. Dry substance and nitrogen content of adrenals of male rats (50–60 g.) treated with a single dose of corticotrophic hormone

No. of animals	Units injected	Time after injection (hr.)	Av. wt. of adrenals (mg.)	% dry substance	% nitrogen of dry substance
5	Controls	—	7·96	28·0	7·68
5	60	1	7·92	28·3	—
5	60	3	7·62	27·1	7·73
5	60	6	8·44	26·3	8·94
5	60	12	8·90	27·2	8·35
5	60	24	9·02	30·2	8·66
5	60	48	8·65	33·2	7·94
5	Controls	—	7·91	33·8	5·47
5	100	3	8·38	32·4	5·01
5	100	6	8·68	28·4	8·08
5	100	12	8·86	33·6	8·17
5	100	24	9·76	35·0	7·07
5	100	48	9·44	36·4	—
5	100	96	9·42	32·4	7·58

Table 3 shows the result of two typical experiments where the dry substance and nitrogen content of the total adrenals of groups of five rats were determined at different time intervals after injection of a single dose of corticotrophic hormone. The percentage dry substance shows a tendency to fall in the first 12 hr. and then to increase up to the 48th hr. The nitrogen content of the dry substance tends to increase continually.

DISCUSSION

Several different processes take place in the adrenal cortex after injection of corticotrophic hormone. Changes in the cholesterol metabolism as a basis for the hormone production, changes in the nitrogen metabolism as a basis for the growth of the organ, and an increase in oxygen consumption all take place. The fact that these different metabolic processes may occur simultaneously may explain the lack of proportionality between the applied dose and the increase in oxygen consumption and also the varying length of the latent period. The increase of oxygen consumption during the first hours after hormone injection might—as in the case of the changing cholesterol content—be due to functional stimulation and hormone production only.

The gain in the adrenal weight during the first hours after a single hormone injection is apparently caused largely by an increasing water content; increase of cholesterol and protein cause the subsequent gain in weight.

The change in the oxygen consumption of the adrenal cortex after injection of corticotrophic hormone is obviously much smaller than that of the ovary after treatment with gonadotrophic hormone (Reiss, Druckrey & Fischl, 1932). The maximum changes observed in the adrenal cortex amounted to about 100% of the normal, whilst the ovary after treatment with gonadotrophic hormone for 24–48 hr. showed changes as high as 400–500%. This difference becomes understandable if the much greater morphogenetic changes in the ovary are borne in mind. The weight of the ovary increases four- to twenty-fold, the weight of the adrenals is hardly doubled.

SUMMARY

1. The respiration and aerobic glycolysis of surviving adrenal cortex have been measured in normal rats and in rats treated with massive doses (50–200 units) of corticotrophic hormone.

2. The adrenal cortex of the treated animals 24 hr. after injection showed a rate of oxygen uptake approximately 25% higher than that of normal rats. This difference proved to be statistically significant.

3. The aerobic glycolysis was rather variable in both the control and experimental series, but occasional high values were noted in the treated group.

4. The water content of the adrenals increased during the first hours after treatment and subsequently decreased.

5. The nitrogen content of the dry substance showed a tendency to rise continuously after treatment.

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MATERNAL AND FOETAL TISSUE- AND PLASMA-LIPIDS IN NORMAL AND CHOLESTEROL-FED RABBITS

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It was previously reported (Barcroft & Popják, 1945) that the plasma-lipid values in the sheep foetus follow closely those of the maternal plasma, but at a lower level. The relationship was so striking that it was suggested that the foetal plasma-lipids in the sheep are dependent upon and probably derived from the lipids of the maternal plasma. A rapid utilization of these substances by the foetus would explain their lower concentration on the foetal side. Boyd & Wilson (1935) showed that the concentration of lipids in the umbilical arterial blood of the mature human foetus is usually lower than in the umbilical venous blood, indicating a utilization by the foetus.

In the investigation to be described the aim was to induce lipaemia in pregnant rabbits and to examine the extent to which the foetal tissue- and plasma-lipids could be influenced by this means.

MATERIAL AND METHODS

For the production of lipaemia in the rabbit a convenient method was available. It was found (Popják, 1945) that the feeding of amorphous cholesterol in a watery suspension by the method of Dömösi & Egyed (1939) caused in a short time an increase of all the plasma-lipid fractions in the rabbit.

Pregnant rabbits of about 3 kg. weight received daily, from the 8th until the 28th day of gestation, 1 g. of amorphous cholesterol. In the initial experiments the watery cholesterol suspension was administered through a stomach tube. Five out of six rabbits thus fed miscarried before the 28th day and the foetuses were lost. These early deliveries can be attributed, at least partly, to the struggle some rabbits make against the passing of a stomach tube. After these failures, therefore, the cholesterol suspension was mixed with a small amount of bran and oats and offered to the rabbits first thing in the morning. The rest of their daily ration was given to them after they had consumed the cholesterol-containing food. Since a small portion of the ration containing the cholesterol was usually spilt from the feeding troughs, it is probable that the actual amount of cholesterol consumed was less than 1 g. per day. The control rabbits were maintained on the same bran, oats and cabbage diet as the experimental ones, only the cholesterol being omitted.

Blood was taken from the experimental animals on the 1st, 8th, 15th and 28th days, and from the controls on the 1st and 28th days, and in some cases on the 15th day of gestation also. Heparin was used to prevent clotting.

On the 28th day the rabbits were anaesthetized by the intravenous injection of nembutal (1 ml. of a 10% solution per 5 lb.). The foetuses were delivered one by one, by the intra-abdominal route, after the uterine horns had been opened along the antimesometrial surface. Blood was drawn from the umbilical vein of each foetus with a small syringe and hypodermic needle. The piston of the syringe was moistened with a small drop of heparin solution. About 1–2 ml. of blood could be obtained from each foetus; the samples were pooled separately from each litter and were heparinized. The differential lipid analyses were carried out on 4–6 ml. of foetal plasma. When the mother bore few foetuses, only 1–2 ml. of foetal plasma could be obtained, and in such cases total cholesterol and total fatty acid alone were determined.

Preparation of tissues for analyses.

All lipid determinations were carried out on tissues dried *in vacuo* from the frozen state over P_2O_5.

Placenta. The placentae were freed of the membranes, wiped with a filter paper to remove adherent blood, then the maternal and foetal portions torn apart. Only the foetal placentae were used for the determinations. The tissues of two to three placentae from each case were pooled. For histological examination whole placentae were taken.

Foetal livers. The whole livers were dissected out from at least two foetuses from each case and pooled. The gall bladder was not removed.

Whole foetuses. The foetuses were minced, dried and ground to a fine powder.

Maternal livers. A sample of about 2–3 g. was excised from the middle of the right lobe.

Lipid analyses.

The plasma was extracted by boiling with at least 20 vol. of an ethanol-ether mixture (3 : 1, v/v) by the method of Bloor (1928). The finely powdered tissues were extracted by boiling with five portions of the ethanol-ether mixture under a reflux condenser.

The filtered extracts were evaporated to dryness under reduced pressure (20–40 mm. of Hg) at an external temperature of 30–40° C. while a slow current of nitrogen was passing through the extract. The dry residues were extracted with light petroleum (b.p. 40–60° C.) and the lipid fractions isolated from this extract. The phospholipids were precipitated and determined by the method of Bloor (1929). For the determination of non-phospholipid fatty acids (derived from neutral fats and cholesteryl esters) a sample of the supernatant fluid obtained after the phospholipid precipitation was evaporated to dryness and saponified with ethanolic KOH (0·2–0·3 ml. of a 40% aqueous KOH solution added to 5 ml. of ethanol) for 2 hr. at 75° C. in an incubator. After completion of hydrolysis most of the ethanol was evaporated off on a hot plate, then about 2 ml. of water were added. The last traces of the alcohol were removed by further heating. The mixture was then acidified with 5% (w/v) HCl and extracted with light petroleum. The fatty acids, redissolved in absolute ethanol, were determined by titration at 75° C. with a 0·02 N-aqueous NaOH solution. The average molecular weight of the fatty acids was assumed to be 275.

Free and total cholesterol were determined by a digitonin method as described by Popják (1943). Ester cholesterol was calculated by difference.

Neutral fats were calculated by the formula:

$$\text{neutral fat} = (\text{non-phospholipid fatty acids} - 0{\cdot}72 \times \text{ester cholesterol}) \times 1{\cdot}05.$$

RESULTS

The results obtained afford a comparison between lipids of (*a*) placentae, (*b*) foetal livers, (*c*) whole foetuses, (*d*) maternal and foetal plasmata, and (*e*) maternal livers of normal and cholesterol-fed rabbits on the 28th day of pregnancy.

Rabbits tolerate very well the administration of amorphous cholesterol in a watery suspension: there is no gastro-intestinal upset as there is when cholesterol is fed in a high fat diet. The only disturbance that cholesterol-feeding (even without a stomach tube) produces in pregnant rabbits is that there is a tendency for premature deliveries, although these are fewer when a stomach tube is not used (three out of nine).

Since some of the results obtained have to be considered in the light of the placental changes brought about by the experimental procedure, these will be described first.

Placentae of cholesterol-fed rabbits

The mean weight of the placentae (foetal + maternal), without the membranes, was approximately the same in normal and in cholesterol-fed rabbits: 6·4 ± 1·4 and 6·3 ± 1·4 g. respectively. The macroscopical appearance of the organ, however, was very different in the two groups of animals (Pl. 1, fig. 1). In normal rabbits the foetal portion of the placenta is of a dark purplish colour and is sharply demarcated from the creamy white maternal part. In cholesterol-fed rabbits the foetal placenta was pale yellow ochre, and it merged indistinctly into the maternal portion.

Histological examination revealed that the paleness of the foetal placenta in the experimental animals was due to the excessive accumulation of lipids in the form of granules within the cells of the foetal capillaries (Pl. 1, fig. 2). Examination of the sections with a polarizing microscope showed that most of this lipid material is anisotropic, i.e. cholesterol and its esters. This histological finding was confirmed by the chemical analyses. The normal foetal placentae on the 28th day of pregnancy did not contain any stainable fatty material, which is in agreement with the description of Chipman (1901–3). Capillary endothelium elsewhere (e.g. kidney) in the body of the mother has an ability also to take up cholesterol, but not nearly to the same extent as the foetal placenta. In the maternal portion of the normal placenta the decidual cells contain stainable fat in the form of distinct globules (Pl. 1, figs. 3, 4). These cells are most obvious around maternal sinuses, arranged in broad sheaths. In cholesterol-fed animals the decidual cells contained far greater quantities of lipids: the fat globules were tightly packed, almost completely obscuring the structure of the cells (Pl. 2, figs. 1, 2). Examination with polarized light showed that the fat in the decidual cells of both control and experimental animals is isotropic, i.e. neutral fat. It is probable that this excessive accumulation of fatty materials in the placentae is the cause of early deliveries by the cholesterol-fed rabbits. It was observed that it was easier to separate the placentae of cholesterol-fed animals from the uterine wall than those of the controls.

The results of lipid determinations on the foetal portions of the placentae (Table 1) show that there was a very marked increase of the free and ester cholesterol, and a moderate increase of the neutral fat content in the cholesterol-fed animals as compared with the controls. The ability of the foetal placenta to accumulate cholesterol and its esters is as great or even greater than that of the maternal liver (cf. Table 9). The mean free and ester cholesterol contents of the experimental placentae were 0·617 ± 0·173 and 2·000 ± 0·686 % respectively, those of the livers of the same animals 0·294 ± 0·074 and 1·290 ± 0·373 % respectively. Considering that these values are expressed as percentage of moist tissue weight and that the water content of the placenta is about 80 % while that of the maternal liver is only about 70 %, the higher cholesterol content of the placentae becomes even more impressive. This behaviour suggests that the placenta is a highly active organ in respect of the fat metabolism of the foetus.

TABLE 1. Lipid content of placentae of normal and cholesterol-fed rabbits on 28th day of pregnancy. Lipid content expressed as g./100 g. fresh tissue

Experiment No.	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters	Total lipid	
			Free	Ester			
61	0·95	1·19	0·226	0·598	0·431	3·395	Placentae of normal rabbits
62	0·62	1·21	0·177	0·195	0·140	2·342	
67	0·59	1·33	0·189	0·246	0·177	2·532	
77	0·54	1·50	0·246	0·430	0·310	3·026	
80	0·44	1·33	0·186	0·141	0·101	2·198	
Mean	0·63	1·31	0·205	0·322	0·232	2·699	
S.D.	±0·19	±0·12	±0·030	±0·190	±0·136	±0·499	
41	1·46	1·25	0·686	2·109	1·576	7·081	Placentae of cholesterol-fed rabbits
64	0·90	1·00	0·650	1·490	1·073	5·113	
65	1·03	1·19	0·598	2·139	1·540	6·497	
71	0·91	1·53	0·491	2·171	1·564	6·666	
78	0·70	2·20	0·952	3·318	2·390	9·560	
79	0·82	1·98	0·513	1·377	0·992	5·682	
81	0·59	1·66	0·431	1·389	1·001	5·071	
Mean	0·92	1·54	0·617	2·000	1·448	6·525	
S.D.	±0·27	±0·44	±0·173	±0·686	±0·496	±1·557	

Lipids of whole foetuses and of foetal livers

The great accumulation of lipids in the placenta apparently interfered with the nutrition of the foetus, if this is judged from foetal body weights. The mean weight of thirty-eight 28-day foetuses from normal mothers was 32 ± 3·6 g., and that of thirty-eight foetuses of the same age from cholesterol-fed rabbits 22 ± 2·2 g. It seems probable that the accumulated lipids blocked the placentae for the passage of nutrient materials. There is further evidence from the lipid analyses of foetal livers and of whole foetuses that in the cholesterol-fed rabbits the nutrition of the foetus was impaired.

The percentage lipid contents of whole foetuses and of foetal livers from normal and cholesterol-fed animals are shown in Tables 2 and 3. It can be seen that the lipid composition of whole foetuses and of the foetal livers differed

Table 2. Lipid content of 28-day foetuses from normal and cholesterol-fed rabbits. Lipid content expressed as g./100 g. dry matter

Experiment No.	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters	Total lipid	
			Free	Ester			
77–1	22·47	3·69	0·507	0·076	0·055	26·80	Foetuses of norma rabbits
77–2	22·53	4·75	0·696	0·104	0·075	28·16	
80–1	22·62	4·76	0·577	0·106	0·076	27·54	
80–2	19·92	4·68	0·602	0·280	0·202	25·68	
80–3	21·69	4·48	0·563	0·296	0·213	27·24	
Mean	21·73	4·47	0·589	0·172	0·124	27·08	
S.D.	±1·10	±0·45	±0·070	±0·110	±0·077	±0·93	
78–1	17·54	5·18	0·586	0·128	0·092	22·53	Foetuses of cholesterol-fed rabbits
78–2	16·36	5·56	0·609	0·104	0·075	22·71	
78–3	14·68	5·37	0·609	0·079	0·057	20·79	
78–4	17·14	5·66	0·681	0·169	0·122	23·77	
79	23·42	4·57	0·672	0·119	0·086	28·87	
Mean	17·83	5·27	0·631	0·120	0·086	23·73	
S.D.	±3·3	±0·43	±0·040	±0·030	±0·024	±3·06	

Table 3. Lipid content of livers of 28-day foetuses from normal and cholesterol-fed rabbits. Lipid content expressed as g./100 g. fresh tissue

Experiment No.	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters	Total lipid	
			Free	Ester			
61	15·40	0·95	0·107	0·123	0·089	16·669	Foetuses of normal rabbits
62	18·06	1·65	0·136	0·054	0·039	19·939	
67	13·50	1·41	0·158	0·089	0·064	15·221	
77	10·07	0·92	0·100	0·126	0·091	11·307	
80	11·46	1·33	0·086	0·120	0·087	13·093	
Mean	13·70	1·252	0·117	0·102	0·074	15·246	
S.D.	±3·17	±0·313	±0·027	±0·031	±0·023	±2·469	
41	10·00	1·45	0·135	0·093	0·067	11·745	Foetuses of cholesterol-fed rabbits
64	8·13	0·97	0·095	0·082	0·059	9·336	
65	6·66	0·97	0·090	0·048	0·035	7·803	
71	11·23	1·78	0·147	0·073	0·053	13·283	
78	8·86	1·68	0·189	0·122	0·088	10·939	
81	17·46	0·80	0·101	0·129	0·093	18·583	
Mean	10·39	1·275	0·126	0·091	0·066	11·948	
S.D.	±3·80	±0·415	±0·038	±0·031	±0·022	±3·766	

Table 4. Average lipid content, in milligrams, of 28-day foetuses from normal and cholesterol-fed rabbits

	Foetuses of	
	Normal mothers (mg.)	Cholesterol-fed mothers (mg.)
Neutral fat	1180	630
Phospholipids	240	185
Free cholesterol	32	22
Ester cholesterol	9	4
Total lipid	1467	844

only in respect of the neutral fat content, this being lower in the cholesterol-fed rabbits. It is of some interest that the foetal livers contained considerably more neutral fat per 100 g. moist tissue than did the maternal livers. Histological examination of the foetal livers (Pl. 2, fig. 3) confirmed the intensive fatty infiltration which, however, was less marked in the foetuses from cholesterol-fed mothers.

If the absolute amounts of lipids contained in the foetuses of the two groups are calculated (Table 4), it is found that the foetuses of cholesterol-fed mothers contained approximately 33% less phospholipid and cholesterol, and approximately 50% less neutral fat, than the controls. The weight of the experimental foetuses was 33% less than that of the controls; therefore it is evident that the phospholipid and cholesterol contents were less by the same proportion as their weights, while the neutral fat content was disproportionately less. When the figures in Tables 1, 2 and 3 are compared, it appears that the neutral fat content of the experimental foetal livers and of whole foetuses is approximately inversely related to the cholesterol content of the placentae. In rabbits Nos. 79 and 81 the placentae differed the least from the normal, as judged by their morphological appearances and cholesterol content. In these two cases the percentage neutral fat content of the whole foetus and of the foetal liver was similar to those of controls, although the foetuses were underweight (22–26 g.). It appears, therefore, that the foetuses of cholesterol-fed mothers are able to build up their tissues with the same phospholipid and cholesterol composition as the controls; when the cholesterol content of the placenta, however, increases beyond a certain point (approximately 1·8% total cholesterol) the foetuses are unable to accumulate the same amounts of neutral fat as the controls either in the liver or in the body as a whole.

The experiments throw very little light on the source of foetal cholesterol. From Tables 2 and 3 it is evident that the percentage cholesterol content of the experimental foetuses and of foetal livers was the same as that of the controls: the absolute amounts (cf. Table 4) were actually less because of the lighter weight of the experimental foetuses. If the foetus obtains its cholesterol from the mother, the controlling mechanism of the placenta must be very exact in this respect, being able to prevent the flooding of the foetus by this compound when the latter is available in excess.

Comparison of maternal and foetal plasma lipids in normal and cholesterol-fed rabbits

Table 5 gives the maternal and foetal plasma-lipid values in the control and experimental animals. These data are represented graphically in Text-fig. 1. It can be seen that normally the concentration of all the lipids is higher in the foetal than in the maternal plasma. Baumann & Holly (1925–6) found that the concentration of lipoid phosphorus and of cholesterol is higher in the blood of

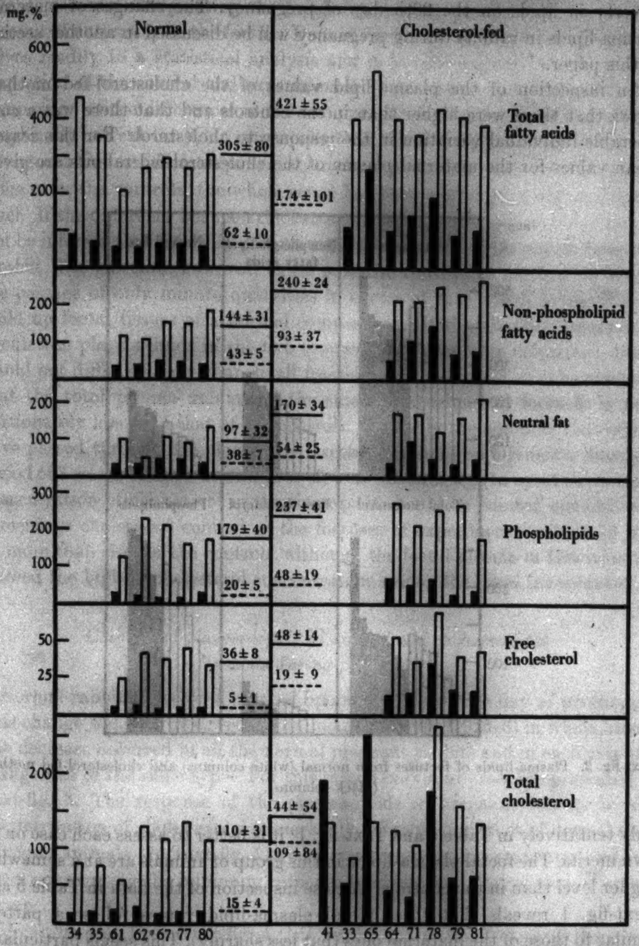
TABLE 5. Maternal and foetal plasma lipids of normal and cholesterol-fed rabbits on 28th day of pregnancy. Lipid content expressed as mg./100 ml. of plasma

Rabbit No.	Total fatty acids		Non-phospholipid fatty acids		Neutral fat		Phospholipids		Free cholesterol		Total cholesterol	
	Mother	Foetus	Mother	Foetus	Mother	Foetus	Mother	Foetus	Mother	Foetus	Mother	Foetus
C34	83	457	—	—	—	—	—	—	—	—	11	80
C35	64	358	—	—	—	—	—	—	—	—	15	78
C61	55	206	38	119	31	100	25	126	5	24	17	57
C62	54	266	40	109	32	49	20	227	6	42	21	130
C67	60	297	50	155	47	105	14	206	4	37	12	113
C77	60	266	48	152	43	90	16	160	5	45	15	136
C80	59	305	41	185	36	139	26	174	5	33	14	111
Mean control	62	305	43	144	38	97	20	179	5	36	15	110
S.D.	±10	±80	±5	±31	±7	±32	±5	±40	±1	±8	±4	±31
33E	110	403	—	—	—	—	—	—	—	—	52	88
41E	375	—	—	—	—	—	—	—	—	—	192	136
65E	267	537	—	—	—	—	—	—	—	—	252	138
64E	101	405	55	218	36	175	68	272	13	53	42	124
71E	146	363	108	217	98	173	48	212	14	36	36	106
78E	195	434	149	257	49	123	66	257	35	70	177	265
79E	90	414	72	238	38	161	26	265	16	40	60	158
81E	106	394	81	272	49	218	35	177	16	43	63	133
Mean experimental	174	421	93	240	54	170	48	237	19	48	109	144
S.D.	±101	±55	±37	±24	±25	±34	±19	±41	±9	±14	±84	±54

rabbit foetuses than in that of the mother. The present investigation confirms the findings of Baumann & Holly and extends their observations to all the lipid fractions of the plasma. The real magnitude of the difference between foetal and maternal values, however, is not so obvious from the work of Baumann & Holly, because they analysed whole blood only. In the guinea-pig also the foetal serum contains more lipids than the maternal (Kreidl & Donath, 1910), while in the human (Slemons & Stander, 1923; Boyd, 1936) and in the sheep (Barcroft & Popják, 1945) the reverse relationship is found. The foetal plasma-lipid values in the rabbit are not only higher than in the mother but, except for the neutral fats, they are even higher than the values in normal adult rabbits (Table 6). The maternal plasma, on the other hand, shows a great

TABLE 6. Comparison between plasma lipid values of 28-day rabbit foetuses and of normal non-pregnant adult rabbits. Lipid content expressed as mg./100 ml. of plasma

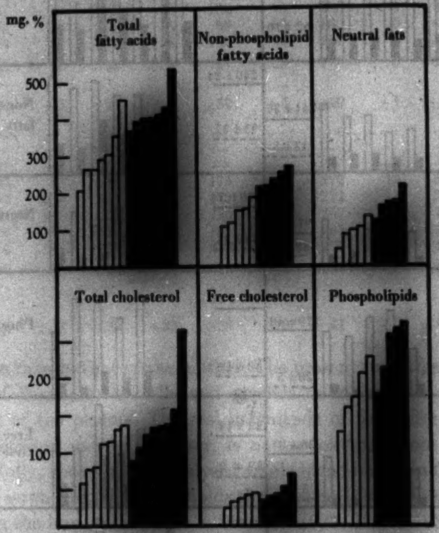
	Total fatty acids	Non-phospholipid fatty acids	Neutral fat	Phospholipids	Cholesterol	
					Free	Tota
Mean of 38 normal non-pregnant adult rabbits	223 ±76	131 ±53	93 ±54	138 ±40	25 ±9	74 ±32
Mean normal foetal values on 28th day of pregnancy	305 ±80	144 ±31	97 ±32	179 ±40	36 ±8	110 ±31



Text-fig. 1. Maternal (black columns) and foetal (white columns) plasma-lipids (mg./100 ml.) in normal and cholesterol-fed rabbits on 28th day of pregnancy. Each pair of columns in the same vertical line represents values for one case whose protocol numbers are given under the columns illustrating total cholesterol. ——— mean foetal, - - - - - - mean maternal values with standard deviation.

poverty in lipids on the 28th day of pregnancy. The changes of maternal plasma-lipids in rabbits during pregnancy will be discussed in another section of this paper.

An inspection of the plasma-lipid values of the cholesterol-fed mothers shows that these were higher than in the controls and that there was a considerable individual variation in the response to cholesterol. For this reason mean values for the maternal plasma of the cholesterol-fed rabbits are given only tentatively in Table 5 and Text-fig. 1; it is better to assess each case on its own merits. The foetal plasma-lipids in this group of animals are at a somewhat higher level than in the controls. A close inspection of the data in Table 5 and Text-fig. 1 reveals that these foetal plasma-lipid values follow a pattern similar to those of the maternal ones (but less sharply). This seems particularly so in the case of total fatty acids, and free and total cholesterol. The usual relationship between maternal and foetal plasma total cholesterol was reversed in experimental rabbits 41 and 65. While it seems that the foetal plasma-lipid values may be influenced to a slight extent by drastic changes in the maternal plasma, the findings in these two rabbits further emphasize the very strict role



Text-fig. 2. Plasma-lipids of foetuses from normal (white columns) and cholesterol-fed mothers (black columns).

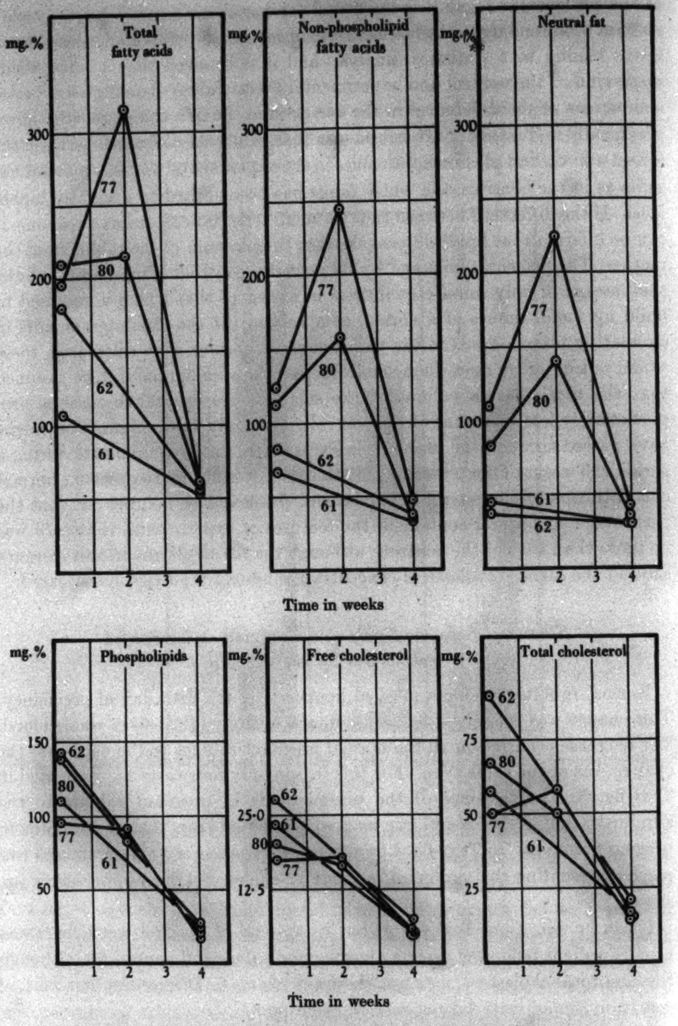
of the placenta in regulating the foetal fat metabolism, providing a certain amount of autonomy to the foetus. Data of such small series do not lend themselves readily to a statistical analysis and it is believed that a side-by-side inspection of the control and experimental foetal values should give a better comparison of the differences in the two groups. Such a comparison is shown graphically in Text-fig. 2. It can be seen that, while the difference between the lowest and highest plasma-lipid values in the experimental animals is about the same as in the controls, the whole range has been shifted to a slightly higher level. If the difference between the two small series is accepted as a real one, it can be inferred that lipids do pass through the placenta of the rabbit from the mother. The placenta, however, by its powerful controlling mechanism allows the passage of only minute quantities in excess of that which is required to build up foetal tissues of a normal composition. If the absolute amounts of circulating plasma-lipids in the two groups of foetuses were calculated, these would not differ by more than small fractions of a milligram, if it be assumed that the total plasma volume of the smaller experimental foetuses is proportionately less than that of the controls. Certainly the amounts that might have passed through the placenta in excess of normal requirements during a period of 3 weeks of intra-uterine growth were not sufficient to upset the normal concentration of cholesterol in the tissues. It should be pointed out that the percentage cholesterol content of the foetuses of experimental rabbit 78 was no more than that of the controls, although the foetal plasma in this instance showed the highest cholesterol concentration among the cases investigated.

Changes of plasma-lipids in normal and cholesterol-fed rabbits during pregnancy

Normal rabbits develop a marked lipopenia by the 28th day of pregnancy. This change was first observed by Baumann & Holly (1925–6) in whole blood. The decrease occurred in all the normal pregnant rabbits and in each case the change was of the same type. For this reason only four cases are presented in Text-fig. 3. The response of the plasma-lipids in pregnant rabbits to the administration of cholesterol was markedly different from that of normal non-pregnant animals. In Text-fig. 4 two cases representing the maximum and two cases representing the minimum response to cholesterol during pregnancy are shown.

It can be seen from Text-fig. 4 that by the end of the first week there was usually a slight fall in the plasma phospholipid values and neutral fat, although free and total cholesterol may not show any changes. During the first week of gestation the animals did not receive cholesterol.

Text-fig. 3 shows that in the normal rabbits by the end of the second week of pregnancy the plasma-lipids generally show a further slight fall, although the



Text-fig. 3. Behaviour of plasma-lipids of normal rabbits during pregnancy. The figures beside the graphs are the protocol numbers of the rabbits.

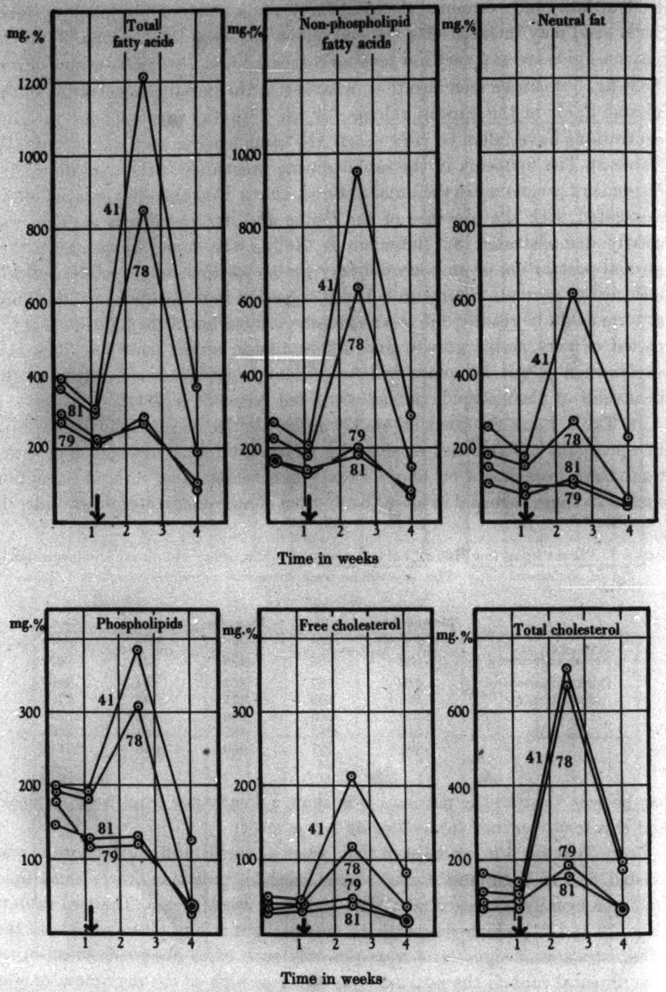
neutral fats (and consequently total fatty acids and non-phospholipid fatty acids also) may increase. By the end of the 4th week (cf. Text-fig. 3) all the plasma-lipids are at a very low level as compared with the initial values. From Text-fig. 3 it can be seen also that, whatever is the initial concentration of the plasma-lipids in the various rabbits, by the 28th day of pregnancy the concentrations have fallen to very nearly the same level in each animal (cf. also Table 5). The lipopenia in the rabbit during pregnancy is the opposite to the lipaemia of pregnancy in the human (Boyd, 1934). That this change is definitely associated with the presence of the foetus and its appendages *in utero* was clearly demonstrated by Baumann & Holly, who found that after either normal parturition or an early delivery the blood lipids of the rabbit quickly returned to normal. Baumann & Holly suggested that the lipopenia of rabbits at term could be readily due to an extensive utilization of the lipids during the period of very active growth and differentiation of the embryos. That this explanation is not adequate will be evident from the consideration of the behaviour of plasma-lipids in cholesterol-fed pregnant rabbits.

In Table 7 are recorded at weekly intervals the plasma-lipid values of a non-pregnant rabbit which received 1 g. of cholesterol per day. Such observations have been made on about twenty-five rabbits and without exception similar changes occurred in all of them, often of an even greater magnitude. It can be seen that all the plasma-lipids show a steady rise from week to week, and this may continue for as long as 2–3 months.

TABLE 7. Plasma lipids (mg./100 ml.) of non-pregnant rabbit before and during administration of 1 g. of cholesterol daily. The interval between successive determinations was 1 week

	Non-phospholipid fatty acids	Neutral fats	Phospholipids	Cholesterol	
				Free	Ester
Before cholesterol	98	66	134	20	35
During cholesterol administration	450	97	361	134	495
	552	109	521	195	615
	1150	266	860	438	1228
	1146	282	835	427	1200
	1261	207	1010	636	1464

From Text-fig. 4 it can be seen that, when administration of cholesterol was started at the beginning of the second week of gestation, the plasma-lipid fractions usually increased to a high level in a week's time. The two rabbits (Nos. 79 and 81) which reacted with only a slight rise in plasma-lipids to the administration of cholesterol were the only such cases observed; in all other experimental rabbits the response was either as high as the maximum or was intermediate between the maximum and minimum changes recorded in Text-fig. 4. By the 28th day, however, a very pronounced fall in plasma-lipids occurred in spite of the continuous administration of cholesterol, but the values



Text-fig. 4. Behaviour of plasma-lipids of cholesterol-fed rabbits during pregnancy. Cholesterol feeding was started at ↓. The figures beside the graphs are the protocol numbers of the rabbits.

had not fallen to such a low level as in the control rabbits. These cholesterol-fed pregnant rabbits showed on the 28th day plasma-lipid values closer to those found initially but, as may be seen from Text-fig. 4, they might be above or below these. That this fall was not due to an inadequate absorption of cholesterol, but to other factors, can be inferred from the fact that it was observed regularly and that the analyses of maternal livers and foetal placentae showed a very marked increase in cholesterol content in every case, proving that cholesterol was adequately absorbed.

That this fall in plasma-lipids was not due to a lack of absorption is further proven by observations on rabbits which miscarried during the administration of cholesterol. The data of a typical case are shown in Table 8. It can be seen that administration of cholesterol for 1 week in the middle of pregnancy produced the usual rise in the plasma-lipids. The animal miscarried on the 26th day; unfortunately no observation was made on the plasma-lipids at that time. Two days later, while the administration of cholesterol was continued, the plasma-lipid values were at an even higher level than in the middle of pregnancy.

TABLE 8. Plasma lipids (mg./100 ml.) of rabbit No. 39 which miscarried on 26th day of pregnancy

Date	Total fatty acids	Non-phospholipid fatty acids	Neutral fat	Phospholipids	Cholesterol	
					Free	Total
23. v. 44, mated	200	100	56	154	28	93
1. vi. 44	236	—	—	—	—	98
2. vi. 44	Cholesterol feeding started					
10. vi. 44	766	589	242	314	210	695
19. vi. 44	Miscarried					
21. vi. 44	914	642	284	394	236	753

It seems that the tendency to lipopenia in rabbits during the second half of pregnancy occurs even when lipaemia is induced experimentally. However attractive the hypothesis of Baumann & Holly, that a high rate of utilization of lipids by the foetus is the explanation for this lipopenia, it seems inadequate. In cholesterol-fed rabbits the amounts of circulating plasma-lipids are far in excess of the requirements of the foetus or of the amounts that are available to it from the maternal plasma during a normal pregnancy. Further, the fall in plasma-lipids was independent of the number of foetuses the mother bore; the plasma-lipids decreased to the same extent whether there were three or ten foetuses present. It seems more likely that a hormonal or some other factor associated with pregnancy is responsible for the suppression of plasma-lipids. An increased fixation by tissues, like that observed in placentae of the cholesterol-fed rabbits, might be more important.

Liver-lipids of normal and cholesterol-fed pregnant rabbits

The relevant data are presented in Table 9. The lipid composition of the livers of control pregnant rabbits on the 28th day of gestation differed from that of normal non-pregnant animals only in respect of the neutral fat content: this is usually not more than 1·5 % of the moist weight in non-pregnant rabbits.

TABLE 9. Lipid content of livers of normal and cholesterol-fed rabbits on 28th day of pregnancy. Lipid content expressed as g./100 g. of fresh tissue

Experiment No.	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters	Total lipid	
			Free	Ester			
61	2·35	2·73	0·143	0·224	0·161	5·608	Normal pregnant rabbits
62	4·38	2·79	0·151	0·101	0·072	7·494	
67	2·95	3·04	0·141	0·089	0·064	6·284	
77	5·17	2·67	0·141	0·336	0·242	8·559	
80	8·24	2·51	0·176	0·150	0·108	11·184	
Mean	4·62	2·75	0·150	0·180	0·129	7·826	
S.D.	±2·313	±0·19	±0·015	±0·102	±0·074	±2·192	
41	2·26	3·06	0·345	1·221	0·880	7·766	Cholesterol-fed pregnant rabbits
64	2·51	2·41	0·211	1·816	1·308	8·255	
65	3·49	2·33	0·263	1·317	0·949	8·349	
71	4·09	2·90	0·281	0·643	0·463	8·377	
78	2·94	3·00	0·428	1·630	1·174	9·172	
79	9·80	2·63	0·296	1·238	0·893	14·857	
81	10·04	3·02	0·231	1·165	0·840	15·296	
Mean	5·02	2·76	0·294	1·290	0·929	10·296	
S.D.	±3·40	±0·31	±0·074	±0·373	±0·277	±3·291	

A moderate degree of infiltration by neutral fat was observed in the control pregnant rabbits. In the cholesterol-fed rabbits, in addition to the infiltration by neutral fat observed in the controls, there was a moderate increase in the free cholesterol, and a marked increase in the cholesteryl ester content. Although rabbits 79 and 81 showed only a slight increase in the plasma-lipids during cholesterol administration, the increased cholesterol content of their livers proves that cholesterol was absorbed. Reference has already been made to this phenomenon.

DISCUSSION AND CONCLUSIONS

The conclusions that can be drawn from the work described are but few and of a tentative character. It seems impossible to influence the foetal plasma-lipids except to a very limited extent by the production of experimental lipaemia in pregnant rabbits. The function of the foetal placenta appears to be of paramount importance in foetal fat metabolism. It is able to prevent the flooding of the foetus by cholesterol and to provide an almost constant milieu for the foetus even under drastic experimental conditions. Boyd (1935) showed that the lipid composition of the placenta varies with the time of gestation: during the last 10 days there is a marked increase in phospholipid content, which suggests an increased physiological activity. He concluded that the passage of lipids through the placenta is not a simple physical transfer but required the active participation of placental tissues. The present experiments substantiate this statement. Although the range of plasma-lipid concentrations in the experimental foetuses was somewhat higher than in the controls, the amounts in excess of the normal were so small that there was no deposition of abnormal amounts of lipids in the foetus. If any appreciable amounts of cholesterol in excess of the normal had passed the placenta, one might have expected to find an increased cholesterol content of the experimental foetuses, since the mechanism to eliminate excess amounts of this compound is particularly sluggish in herbivorous animals. Unless the rabbit foetus has a great ability to destroy excess cholesterol, it can be concluded that only very small, almost negligible, amounts of cholesterol in excess of the normal pass through the placenta. This conclusion of course does not exclude the possibility that the normal cholesterol requirements of the foetus are derived from the preformed maternal compound.

While there is evidence from the works of Sinclair (1933), Chaikoff & Robinson (1933), McConnell & Sinclair (1937) on the rat, and of Bickenbach & Rupp (1931) and Rupp & Bickenbach (1931–2) on the rabbit, that fatty acids (or their simple compounds) do pass through the placenta from the mother to the foetus, there has been very little done to investigate with modern methods the possibility of foetal synthesis of fats. The recent investigations of Nielson (1941–2) on the rat with radioactive phosphorus show that probably the major portion, if not all, of the foetal phospholipids are synthesized within the foetus and are not derived from the mother. The higher plasma-phospholipid content of the experimental foetuses in the present experiments may not be due to an excess transfer of this compound through the placenta but may have been synthesized in larger amounts within the foetus or placenta, owing to the abnormal condition of the latter. It is well, however, to bear in mind the species differences. There are two alternative explanations for the higher lipid content of the foetal plasma in the rabbit as compared with the maternal: (*a*) that the placenta in this species has a power to concentrate lipids, assuming that the foetal lipids are derived from the mother; or (*b*) that there is a synthesis of lipids within the foetus in addition to those obtained from the mother. The second alternative is favoured, as further discussion will show.

The placentae of the cholesterol-fed rabbits contained large quantities of free cholesterol and cholesteryl esters and also some excess neutral fat. This abnormal lipoid storage apparently produced a pathological condition resulting in an impaired foetal nutrition, if this is judged from body weight. Not only were the experimental foetuses 33% lighter than the controls, but they contained also less neutral fat, although the concentration of the other lipids was similar to that in the controls. If all the foetal fat were derived from preformed maternal fat, it is difficult to understand why the fatty acids should not reach the foetus, at least in normal amounts, when they are available in excess within the placenta. The results are most readily interpreted if it be assumed that preformed maternal fat is not the only source of foetal fat but that some of it may be synthesized within the foetus. If this hypothesis be accepted, until further evidence on the question is obtained, the lower neutral fat content of the experimental foetuses may be explained on the basis that the placentae were partially blocked by the accumulated cholesterol and thus limited the free passage of fat precursors from which the foetus could synthesize fat. Boyd (1935) showed that during the last 8–10 days of gestation there is a sudden marked increase in the neutral fat content of the rabbit foetus. If all this neutral fat is derived from the mother, there must be a sudden increase in placental permeability to it. It was pointed out by Huggett (1941), in his review on foetal nutrition, that, owing to degenerative changes, the permeability of the placenta may increase towards the end of gestation. One cannot exclude the possibility, however, that by the 20th–22nd day of the rabbit's gestation the embryonic tissues reach such a degree of maturation that the enzyme systems required for fat-synthesis from carbohydrates may operate. Further investigation of this problem with the aid of isotopes should answer many as yet unsolved questions of foetal fat metabolism.

The lipopenia observed in pregnant rabbits towards the end of pregnancy may be due to hormonal or other factors associated with gestation. The explanation of Baumann & Holly that an increased utilization of maternal lipids by the foetus is the cause of the lipopenia seems inadequate, because (*a*) the same phenomenon was also observed in all the cholesterol-fed rabbits in which the foetuses actually utilized less lipids than did the controls, and (*b*) because the degree of lipopenia was independent of the number of foetuses the mother bore.

SUMMARY

1. Lipid analyses were carried out on the placentae, foetal livers, whole foetuses, maternal livers, and maternal and foetal plasmata of normal and of cholesterol-fed rabbits, on the 28th day of pregnancy.

2. Feeding of amorphous cholesterol to pregnant rabbits during the last 3 weeks of gestation caused a heavy storage of free and esterified cholesterol and an increase of neutral fat content in the foetal placenta. From histological investigations it is inferred that there was also an increase of neutral fats in the decidual cells of the maternal portion of the placenta.

3. The lipoid-storage in the placenta interfered with the nutrition of the foetus, resulting in foetuses one-third lighter than normal. These foetuses and their livers contained less neutral fat than the controls.

4. It is suggested that not all the foetal fat is derived from preformed maternal fat but that at least a part of it may be synthesized within the foetus.

It is inferred, on the basis of this assumption, that the lower neutral fat content of the experimental foetuses was brought about by the partial blockage of the placentae to the transfer of fat-precursors.

5. The concentration of lipids was considerably higher in the foetal than in the maternal plasma, and was even higher than in normal non-pregnant adults of this species. It was impossible to influence the foetal plasma-lipid values except to a very limited extent by the production of lipaemia in the mothers.

6. The rabbit develops a very marked lipopenia by the end of gestation, in contrast to the lipaemia of pregnancy in the human. Although rabbits usually responded to cholesterol-feeding with a marked lipaemia in the middle of gestation, by the 28th day a tendency towards lipopenia manifested itself. When a cholesterol-fed animal miscarried, there was an immediate rise in plasma-lipids. It is suggested that a hormonal factor, or an increased fixation of lipids by the tissues, may be the cause of the lipopenia.

I gratefully acknowledge my indebtedness to Sir Joseph Barcroft, with whom the first operations were performed and who has given valuable advice throughout these experiments. My thanks are also due to Miss M. Kendall for her technical assistance and to Mr A. E. Clark for the preparation of the histological sections, which, particularly in the case of frozen sections from placentae, was no mean task.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Appearance of placentae from normal (A) and cholesterol-fed rabbits (B) on 28th day of pregnancy. The cross-section of each is also shown. Approximately ½ original size.

Fig. 2. Photomicrograph from the foetal portion of placenta of cholesterol-fed rabbit No. 64. Section cut on freezing microtome and stained with Scharlach R alone; all the black material represents lipids within cells of foetal capillaries. Most of these lipids are anisotropic. 93 ×.

Fig. 3. Photomicrograph from the maternal portion of placenta of control rabbit No. 80. Section stained with Scharlach R and haematoxylin. Picture shows decidual cells containing fat granules and arranged around maternal blood sinuses. All the fat in these cells is isotropic. 80 ×.

Fig. 4. Photomicrograph from the same field as fig. 3; it shows isolated decidual cells containing distinct fat granules. 480 ×.

Plate 2

Fig. 1. Photomicrograph from maternal portion of placenta from cholesterol-fed rabbit No. 41. The area represented here corresponds to that shown from a control rabbit in Pl. 1, fig. 4. The decidual cells contain large amounts of isotropic fat. Section stained with Scharlach R and haematoxylin. 42 ×.

Fig. 2. Photomicrograph from the maternal portion of placenta from cholesterol-fed rabbit No. 33. It shows decidual cells loaded with isotropic fat and arranged around maternal sinuses. The individual structure of the cells has been completely obscured by fat. 80 ×.

Fig. 3. Photomicrograph from normal foetal liver (28-day foetus of rabbit No. 80). Section stained with Scharlach R and haematoxylin. The liver cells contain large globules of fat. 480 ×.

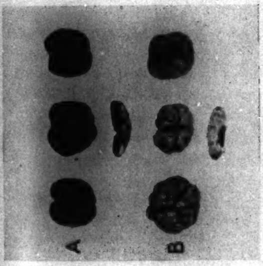

Fig. 1.

Fig. 2.

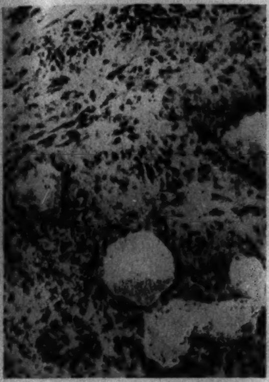

Fig. 3.

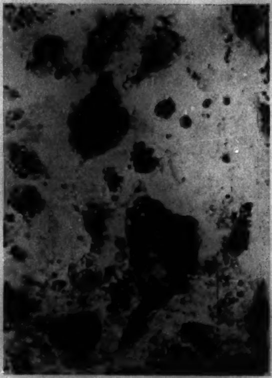

Fig. 4.

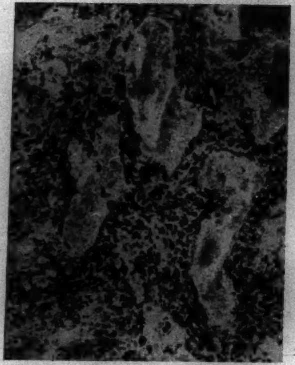

Fig. 1.

Fig. 2.

Fig. 3.

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ON THE ACTION OF ADRENALINE ON THE BLOOD VESSELS IN HUMAN SKELETAL MUSCLE

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Grant & Pearson (1938) found that adrenaline in small doses regularly caused vasodilatation in human muscle. Holling (1939) showed that its action was not quite so straightforward as Grant & Pearson's observation implied. When adrenaline was infused intravenously the vasodilatation was only transient and passed off in a few minutes although the infusion continued. This interesting observation was mentioned incidentally, since Holling was concerned with another problem.

The object of this paper is to give a more detailed account of the action of adrenaline on the blood vessels in human muscle.

METHODS

Healthy men aged 20–30 yr. acted as subjects. Room temperature was about 20° C.

Intravenous infusion of adrenaline. The subject stripped to the waist and lay with his back raised to an angle of about 45°. He was made comfortably warm with blankets. The left forearm was shaved. The plethysmograph for measuring the blood flow by Lewis & Grant's method was fitted on (Barcroft & Edholm, 1943, 1945). Water-bath temperature was 34° C. (Barcroft & Edholm, 1946). The arrangements for recording were completed and the apparatus was calibrated.

The skin over the antecubital vein of the right arm was anaesthetized with procaine. A sphygmomanometer cuff was put on the upper arm and inflated to about 80 mm. Hg. The subject was asked to open and close his hand several times to fill the veins. A sterile hypodermic needle about 1·5 mm. in diameter was fitted to a syringe and introduced some distance into the vein. As soon as blood entered the syringe the pressure in the sphygmomanometer cuff was released. The syringe was taken off and the apparatus shown in Fig. 1*a*, filled with sterile saline, was plugged into the intravenous needle. A few c.c. were run in from the burette to wash out the needle, and the stream was reduced to a trickle. The needle was fixed to the skin with sticking plaster.

Records of the blood flow in the forearm were made until a steady flow was obtained (usually in about 15–20 min.). A 50 c.c. syringe was filled with saline containing the desired concentration of adrenaline, placed in the apparatus shown in Fig. 2, and connected to the side limb of the T-piece (Fig. 1*a*). Then recording began at ½ min. intervals, and after about 1 min. the adrenaline infusion was started. It continued for 10 min., during which time the condition of the subject's complexion and breathing was noted. Recording was continued for about ½ hr. after. Most experiments then ended. In some a second infusion was given.

In some experiments the plethysmograph was used for recording the blood flow in the hand (Barcroft & Edholm, 1945).

Experiments were also done on sympathectomized subjects. Landis & Gibbon's (1933) body-heating and finger-temperature test, and the body-heating and forearm-blood-flow test (Wilkins & Eichna, 1941) were used to ascertain that the sympathectomy had been complete.

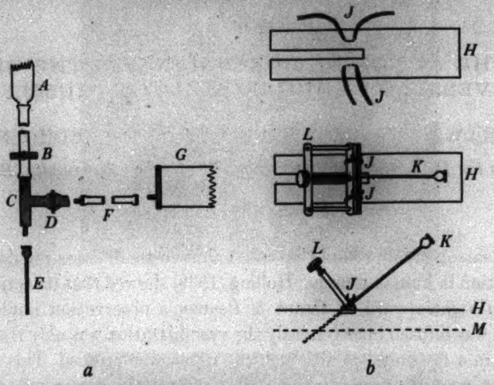

Fig. 1. (*a*) *A*, 25 c.c. burette; *B*, screw-clip; *C*, metal T-piece; *D*, side limb and tap; *E*, intravenous or intra-arterial needle; *F*, connecting piece; *G*, 50 c.c. syringe. (*b*) *H*, adhesive plaster 10 × 2½ cm. with piece cut out as shown; *J*, *J*, strings; *K*, intra-arterial needle; *L*, screw-clip; *M*, femoral artery.

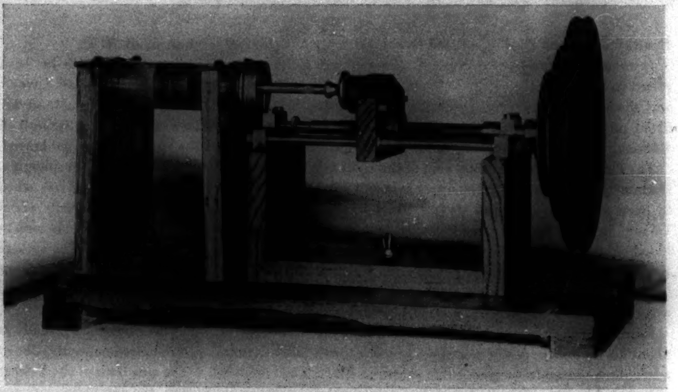

Fig. 2. The infusion apparatus was driven by a $\frac{1}{30}$ h.p. motor and reduction gear. The fluid left the syringe at the rate of 4 c.c./min.

Intra-arterial infusion of adrenaline. Adrenaline was infused intra-arterially in order to avoid its action upon the heart and to study its direct effect on the peripheral circulation. The combined techniques of arterial puncture (Peters & Van Slyke, 1932) and blood-flow measurement were found to be more easily applied to the leg than to the arm. The procedure was as follows.

The subject lay flat on his back so that the femoral artery was stretched. A stand for the burette was placed between his legs as near to his body as possible. A plethysmograph was fitted to the calf and filled with water maintained at 33–34° C. The venous and arterial occlusion cuffs were put on, and the arrangements for recording the calf blood flow were completed.

Poupart's ligament was located, and the course of the femoral artery for several inches below was very carefully palpated and marked on the skin. In this one was aided by watching the movement of the finger caused by the arterial pulsation. If the finger was slightly to one side of the vessel the arterial pulse pushed it sideways. Having drawn the line over the artery, a spot 2–5 cm. below the ligament, where the pulsations were most easily felt, was marked for puncture. The surrounding skin was shaved. A piece of adhesive plaster previously prepared as shown in Fig. 1*b* was stuck on with the two ends towards the toes, the space between the ends was exactly over the line of the artery, and the strings were about 1 cm. proximal to the spot chosen for puncture. The skin between the ends was cleaned and infiltrated with procaine.

The top of the burette (Fig. 1*a*) was connected to the air reservoir for the arterial occlusion cuff so that the saline was under about 200 mm. Hg pressure. A small syringe containing procaine was fitted to the side tube (Fig. 1*a*). A hypodermic needle 0·8–0·9 mm. external diameter and about 6 cm. long was fitted on to the adapter. The screw-clip, shown in Fig. 1*b* was slipped over the needle. Two people were necessary for inserting the needle into the artery. One pierced the skin in the space between the ends of the plaster, at the level of the strings, pointing the needle towards the toes at an angle of about 45°. While this one held the needle steady, the other injected a little procaine into the subcutaneous tissues. Then the needle was pushed in slowly, with several pauses for the infiltration of more procaine until arterial blood could be withdrawn. As soon as the needle was in the artery it was pushed in another 1 cm. (This technique succeeded at the first attempt in six out of eight subjects; in one the artery was missed, or possibly went into a spasm, so the needle was withdrawn; in another the procaine failed to anaesthetize the subcutaneous tissue adequately, and the experiment was stopped.) Owing to the care taken to ensure proper anaesthesia four of the six subjects did not feel the needle entering the artery, the other two, on being questioned later were dimly conscious of a little pain. (If the arterial wall is not anaesthetized fainting may occur when it is pierced (Rushmer, 1944).) The procaine syringe was then replaced by one containing saline, the side tube and needle were flushed out, the tap closed and a slow trickle of saline from the burette started. To hold the intra-arterial needle in position the screw-clip was tightened round it near the skin and tied to the plaster with string (Fig. 1*b*). The angle of the needle was kept about 45° or somewhat less by adjusting the height of the burette.

Calf blood flows were recorded for about 15 min. Records were then made every ½ min. and adrenaline infused for about 5 min. After a further 5 min. of recording, making about ½ hr. altogether, the intra-arterial needle was taken out, pressure being applied over the puncture point for a few min. There were no undesirable after-effects.

RESULTS

Intravenous infusion. Fig. 3 shows the results of six consecutive experiments, each on a different subject. In all experiments adrenaline was infused intravenously at 10 μg./min., and the heart rate quickened and then slowed during the first 2 min. of the infusion (see Hume, 1927). Thereafter it was above the resting rate in five experiments and below it in one (No. 5). In all experiments the forearm blood flow, like the heart-rate, quickened and then slowed during the first 2 min. This confirmed Holling (1939). During the remainder of the infusion the blood flow was above the resting level in all experiments, though not very much so in Exp. 5. It is worth noting that in two experiments, Nos. 2 and 6, a slight decrease in flow, like that observed by Holling, took place at the very beginning of the infusion, and also that in Nos. 3 and 5, after the 'peak', the flow decreased to a minimum, which in Exp. 5 was actually less than the resting rate. The averages of the heart-rates and forearm flows of these six experiments are shown in the lower part of Fig. 4; the likeness between the two curves is remarkable.

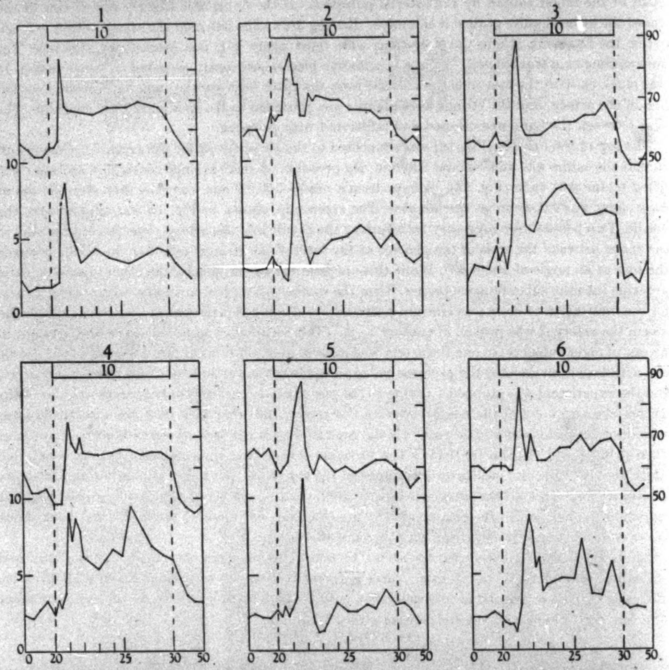

Fig. 3. Between the dotted vertical lines adrenaline was infused intravenously. The rate, in μg./min., is given under the upper margin of each diagram. Upper curve: heart-rate in beats/min. Lower curve: blood flow in the normal forearm in c.c./100 c.c. forearm/min. Time, min.

Fig. 5 shows a typical tracing recorded during the first 4 min. of an infusion of 10 μg. of adrenaline per min. The heart beats cannot be seen distinctly owing to the reduction of the original to one-fifth in copying. The figure shows the sudden increase and then decrease of the forearm flow, with a similar change in the forearm volume (see also Grant & Pearson, 1938).

The effects of infusions of smaller and of greater concentrations were tried. Fig. 6 shows three experiments, in which the concentration was 1 μg./min. The heart was almost unaffected, but in Nos. 2 and 3 the forearm flow showed the

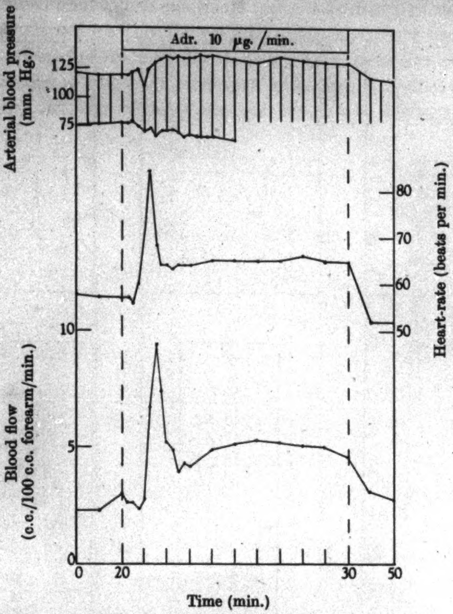

Fig. 4. Averaged results of the experiments shown in Fig. 3.

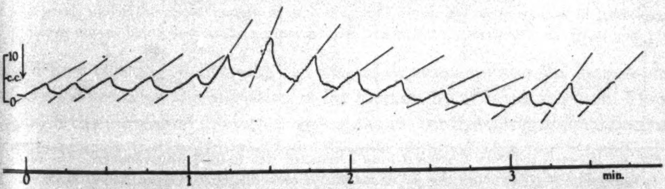

Fig. 5. Tracing of the blood flow in the normal forearm obtained by Lewis & Grant's plethysmographic method. Intravenous infusion of adrenaline was begun at the arrow at 10 μg./min.

typical initial quickening and slowing. Fig. 6 also shows three experiments in which the concentration was 20 μg./min. The general picture in Exps. 4 and 6 resembles that seen with the 10 μg. infusions. The subject of Exp. 5 was known to be unusually sensitive to adrenaline, and 2 min. after the start of the infusion the neck muscles began to contract spasmodically, so, at the end of the third minute, the infusion was stopped. Nevertheless, by this time the conspicuous quickening of the heart-rate and of the forearm blood flow was over and marked slowing had occurred.

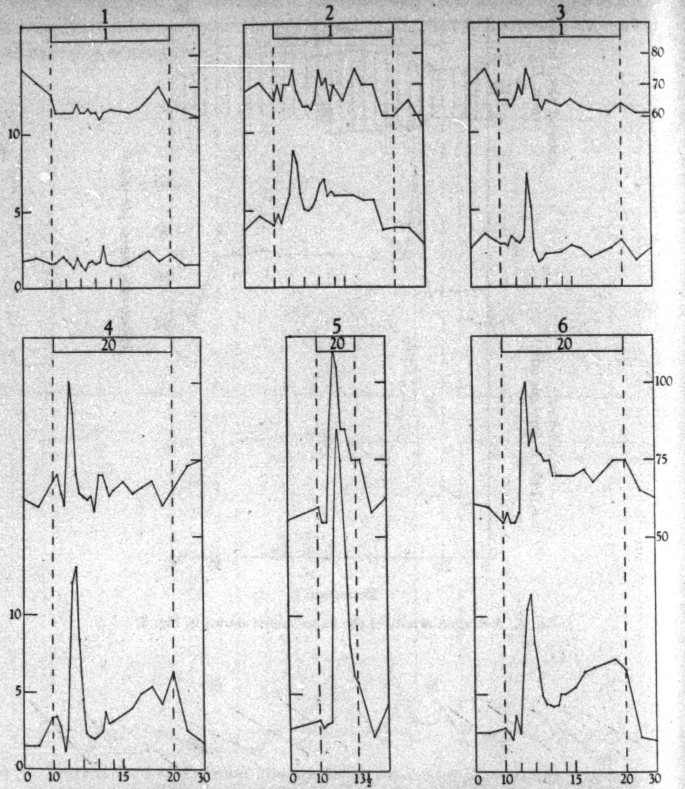

Fig. 6. Between the dotted vertical lines adrenaline was infused intravenously. The rate, in μg./min., is given under the upper margin of each diagram. Upper curve: heart-rate in beats/min. Lower curve: blood flow in the normal forearm in c.c./100 c.c. forearm/min. Time, min.

The cause of the early vascular changes was now sought. It is unlikely that they were due to emotional excitement, since, in a control experiment, the subject thought he was receiving an adrenaline infusion, but the syringe actually only contained saline. This had no effect on the heart-rate or forearm blood flow. The next infusion, containing adrenaline, caused the typical changes described above. The emotional factor was therefore ruled out.

We next considered whether the vascular changes could be secondary to the action of the adrenaline upon the heart. This seemed unlikely for the following reasons. Infusions of 1 μg./min., too weak to have any significant effect upon the rate of the heart, caused the typical quickening and slowing of the forearm blood flow (see Fig. 6, Exp. 2). In several experiments also the increase in heart-rate preceded the dilatation in the forearm by as much as ¼ min. Then again, if the peripheral dilatation were a passive cardiac effect it should occur simultaneously in the arm and leg. Records obtained in a few experiments showed, however, that it appeared in the forearm about ¼ min. before it did in the leg. Further evidence against the cardiac origin of the early peripheral vascular changes is shown in Fig. 7. Each subject had had 2 mg. atropine sulphate intramuscularly about ½ hr. before the adrenaline infusion. During the infusions, cardiac acceleration took place in Nos. 1 and 3 but the characteristic slowing did not occur. Its absence in No. 3 is noteworthy. Yet in all subjects quickening of the forearm blood flow was followed by the typical

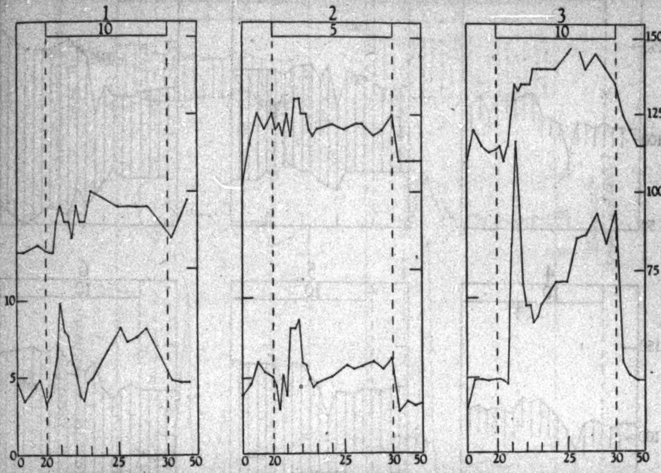

Fig. 7. Each subject given 2 mg. atropine sulphate intramuscularly about ½ hr. before the infusion. Between the dotted vertical lines adrenaline was infused intravenously. The rate, in μg./min., is given under the upper margin of each diagram. Upper curve: heart-rate in beats/min. Lower curve: blood flow in the normal forearm in c.c./100 c.c. forearm/min. Time, min.

slowing. Furthermore, Wiggers & Werle (1942) state that in passive vascular changes the rate of the blood flow is directly proportional to the mean arterial blood pressure. Fig. 4 shows that soon after the beginning of infusion the blood flow increased, on the average, about fourfold. If this was a passive effect the mean arterial blood pressure must have increased about fourfold, i.e. to about 400 mm. Hg! Fig. 8 shows six experiments in which the arterial blood pressure

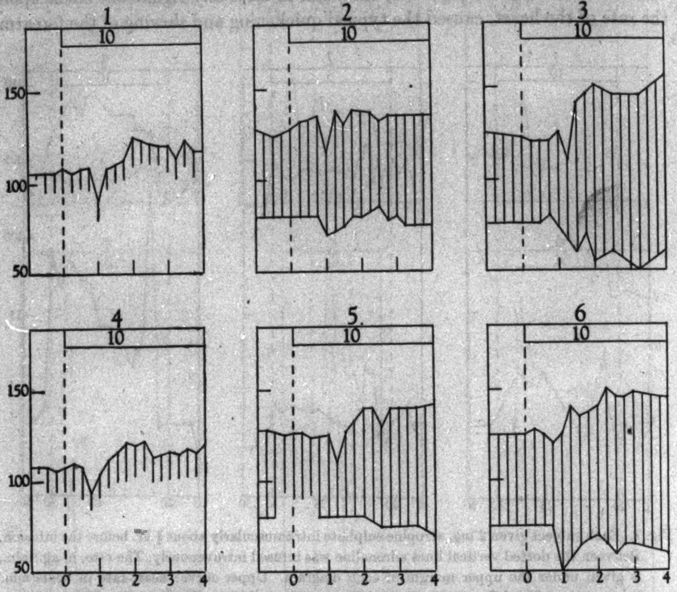

Fig. 8. Adrenaline was infused intravenously beginning at the vertical dotted line. The rate, in μg./min., is given under the upper margin of each diagram. Ordinates: arterial blood pressure in mm. Hg. Abscissae: time, min.

was measured at $\frac{1}{2}$ min. intervals during the infusion of adrenaline at 10μg./min. There is no sign of a prodigious rise of arterial pressure; on the contrary, a transitory but distinct decrease of blood pressure is the first change disclosed (see Gordon & Levitt, 1935). Later there is a rise in systolic and a fall in diastolic pressure without a great change in the mean arterial blood pressure (see also the averages of these blood-pressure results shown in the upper part of Fig. 4). These findings, too, deny the cardiac origin of the vascular effects seen in the forearm. On the other hand, the fall in the blood pressure might well have been due to active vasodilatation in the peripheral vascular system.

Intra-arterial infusion. It was at this point that it was decided to perform the crucial experiment of infusing adrenaline into the femoral artery in order to avoid its action on the heart and to observe its direct effect upon the circulation in the limb. Fig. 9 shows two such experiments. The rate of infusion was 2–3 μg./min. Soon after the infusion began there was a 5–6-fold increase in the calf blood flow. This must have been due to active vasodilatation since the heart-rate and arterial blood pressure remained constant. The trivial increase in heart-rate in Exp. 2 was probably due to the vasodilatation in the leg and loss of blood from the general circulation. Asmussen, Christensen & Nielsen (1939), and Jarisch & Gaisböck (1929) found that an increase in the amount of blood in the legs is followed by immediate reflex cardiac acceleration with no perceptible change in arterial blood pressure.

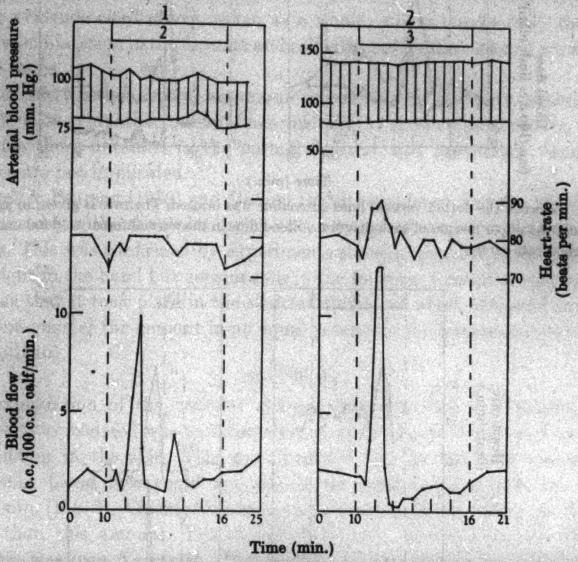

Fig. 9. Between the dotted vertical lines adrenaline was infused intra-arterially. The rate, in μg./min., is given under the upper margin of each diagram.

Finally, there is the experiment shown in Fig. 10 in which the blood flow in the forearm was measured as well as that in the calf. The facts appear to admit of the following explanation only. Soon after the intravenous infusion began the adrenaline stimulated the heart and also excited vasodilatation in the forearm. A little later it did the same in the calf. In the arterial infusion the

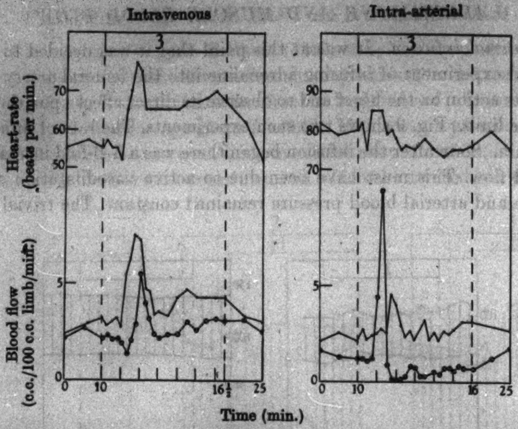

Fig. 10. Between the dotted vertical lines adrenaline was infused. The rate is given, in μg./min., under the upper margin of each diagram. Blood flow in the normal forearm (dots) and in the normal calf (circles).

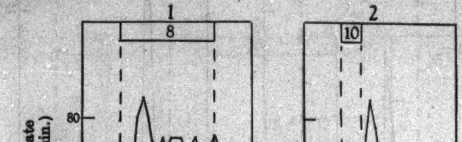

Fig. 11. Between the dotted lines adrenaline was infused intravenously. The rate, in μg./min., is given under the upper margin of each diagram.

whole of the adrenaline entered the femoral artery. Its first effect was a very large dilatation in the calf. This occurred before it reached the heart, so there was no increase in the heart rate or in the forearm blood flow (the trivial increase of about 5 beats/min. was probably reflex in origin, see above). Most of the adrenaline was absorbed into the tissues of the leg, but a little remained in the venous blood. It was not enough to stimulate the sino-auricular node, but it caused a little vasodilatation in the forearm ¾ min. after the vasodilatation in the leg.

The evidence cited above, taken as a whole, proves conclusively that the early vasodilatation in the forearm and calf is caused by the action of adrenaline in the limbs.

To decide whether central vasomotor reflexes played any part, experiments were done on sympathectomized forearms. Fig. 11 shows typical results, which resemble those obtained in the normal forearm, and prove that vasomotor reflexes are not implicated.

Grant & Pearson (1938) and Holling (1939) considered that the vasodilatation must be in the skeletal muscles since adrenaline constricts the cutaneous vessels. This was confirmed by experiments showing that the early dilatation was slight in the hand but conspicuous in the forearm, a result according with the idea that it took place in the skeletal muscle, of which the hand contains only one-quarter the amount in an equal volume of the forearm (Abramson & Ferris, 1940).

DISCUSSION

The explanation of the vascular changes just described is unknown. One possibility considered was vasodilatation in skeletal muscle followed by vasoconstriction in the skin. This was discarded for the following reason: the cutaneous blood flow could not exceed the total forearm flow, i.e. about 3 c.c./min. (Fig. 4). Closing the cutaneous vessels could not reduce the flow by more than this amount. Yet, during the 'peak' constriction, the average decrease was over 5 c.c./min. (Fig. 4). This implies that both dilatation and constriction took place in the same vascular bed, i.e. in the muscle.

The results of experiments on animals are suggestive. The literature is summarized by McDowall (1938). Dale & Richards (1918), Clark (1933) and Roome (1938) found that, under certain conditions adrenaline caused vasodilatation followed by vasoconstriction in skeletal muscle. There was strong evidence that the dilatation was in the capillaries and the constriction in the arterioles. Hartman, Evans & Walker (1928) studied the blood vessels in cat's muscle under the microscope during intravenous injection of adrenaline. In some experiments the capillaries dilated and the arterioles constricted. Possibly the microscope might solve the question in man.

There are sympathetic vasodilator and vasoconstrictor fibres in human muscle (Barcroft, Bonnar, Edholm & Effron, 1943; Barcroft & Edholm, 1945).

The nature of their chemical transmitters is unknown. No light is thrown on this question by the experiments described above. In the dog, adrenaline causes vasodilatation in muscle, but the dilator fibres are cholinergic (Bülbring & Burn, 1934). In man adrenaline causes vasodilatation, but this is no proof that the vasodilator fibres are adrenergic.

Since the rate of adrenaline secretion by the active human suprarenal glands is unkown, it is not possible to say whether the rates of intravenous infusion used were 'physiological'. Most of the subjects experienced palpitation at the beginning of the infusions, but not more than sometimes happened naturally.

The sustained vasodilatation which follows the 'peak' in our experiments also follows subcutaneous injection of adrenaline (Kunkel, Stead & Weiss, 1939). Its cause requires further investigation.

SUMMARY

1. Adrenaline was infused intravenously and intra-arterially. Heart-rate, arterial blood pressure, and blood flow in the forearm, calf and hand were studied in normal and sympathectomized subjects.

2. During intravenous infusion at 10 μg./min. for 10 min. the blood flow in the forearm:

(*a*) increased 4–5-fold in the first 2 min.;
(*b*) subsided to twice the resting rate during the third min.;
(*c*) remained constant till the end of the infusion.

3. Qualitatively similar changes took place in the heart-rate.

4. With reference to the initial vasodilatation (2*a*) and constriction (2*b*):

(i) Evidence is submitted proving that they were not cardiac in origin but were due to the action of the adrenaline in the limb. For example, a similar change in the blood flow in the calf took place during infusion of adrenaline into the femoral artery, although the heart rate and arterial blood pressure remained constant.

(ii) They occurred in sympathectomized limbs. Central vasomotor reflexes were not involved.

(iii) They were much less conspicuous in the hand than in the forearm, showing that they took place in the skeletal muscles.

(iv) Animal experiments suggest that they may be due to capillary dilatation followed by arteriolar constriction.

We express our deep appreciation of the help given by the members of the Belfast Medical Students' Association and by the sympathectomized subjects. We thank Mr Loughridge and Mr Purce for putting us in touch with the sympathectomized subjects; Dr G. T. C. Hamilton for assistance and Mr Wylie, Superintendent of the Physics Workshop of the University, for technical assistance. The expenses were defrayed by the Medical Research Council.

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THE EXPERIMENTAL PRODUCTION OF WATERY VACUOLATION OF THE LIVER

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In this paper it will be shown that when the oxygen supply to the liver is seriously impaired, water passes from the blood into the liver cells and forms large watery vacuoles in the cell cytoplasm. This phenomenon only occurs provided that, during the period of liver anoxia, the blood pressure in the liver sinusoids is maintained at least at its normal value; if the pressure is above normal, the liver-cell vacuolation is more rapid and greater in extent. It seems that anoxia, by increasing the permeability of either the sinusoids or the liver-cell walls, allows the existing hydrostatic pressure in the sinusoids to force water, and probably other plasma constituents, into the liver cells.

It is necessary to distinguish two sets of circumstances under which this liver-cell vacuolation has been found to occur.

(i) *In vivo vacuolation.* Vacuoles may develop during life in any conditions of severe anoxia. The anoxia must be very severe, to the point of threatening life, and must persist for 20–30 min. before significant vacuolation occurs.

(ii) *Post-mortem vacuolation.* When an animal dies from anoxia (or asphyxia) vacuoles rapidly develop during the first 5–15 min. post-mortem, provided the animal is not bled out. In such an animal the venous blood pressure rises considerably just before death and a positive venous pressure persists for some time after death; this pressure is communicated back to the liver sinusoids and is sufficient to cause vacuolation of the highly anoxic post-mortem liver. In death from other causes, the intrahepatic blood pressure after death is approximately zero and so no vacuoles develop.

One proviso must be made with regard to the above statements—the liver must contain no more than a moderate amount of glycogen. If the liver is full of glycogen vacuolation will usually not occur under the conditions specified.

HISTOLOGICAL DESCRIPTION

This description applies to the rat, rabbit, guinea-pig and monkey. The vacuoles referred to are present in the liver-cell cytoplasm. In size they range from 2 to 12 μ; most commonly they are about the size of the nucleus or rather less (Pl. 1, figs. 6–11). The number in each cell ranges from 1 to about 12, most commonly it is 2 or 3. While in general their shape is spherical, many of them are definitely 'off round', being ovoid or slightly polyhedral or flattened on one side (Pl. 1, figs. 10, 11). When there are only a few vacuoles in the cell they tend to be located centrally, close to the nucleus. Often the nucleus indents the vacuole which then appears as a crescent closely applied to one side of the nucleus. Less commonly the vacuole may indent the nucleus and the nucleus appears as a crescent applied to the vacuole. This suggests that the intravacuolar pressure is very variable. Occasionally a small vacuole has been seen lying wholly within the nucleus.

The vacuoles are clearly visible in fresh living liver cells mounted in heparinized plasma from the same animal. In the living cell the cytoplasm appears granular and usually contains some small highly refractile fat droplets. The nucleus appears as a pale grey homogeneous area bounded by a membrane and containing a nucleolus. The vacuoles appear as pale grey non-granular areas in the cytoplasm with smooth regular margins. They look very like the nucleus, and, when of comparable size, can only be distinguished by the absence of a nucleolus. If the cells are ruptured by gentle pressure, the granular cytoplasm may break away in fragments leaving the vacuoles as discrete bodies floating freely in the plasma and the vacuoles are then seen to be bounded by a definite membrane. They have no power of segregating neutral red, methylene blue or methyl green. Their optical properties (translucence, refraction) indicate definitely that they are, in fact, watery vacuoles; they are certainly neither gaseous nor lipoid, and their appearance in paraffin sections rules out any question of their being solid. The slightly irregular shape of the vacuoles, already referred to, is equally apparent in the living cell, and so it does not represent a fixation artifact. Nor is it due to mutual pressure of adjacent vacuoles, for a solitary vacuole lying well away from the nucleus may also be irregular in shape. This is interesting as it suggests the presence of an invisible structural architecture in the cell cytoplasm.

In paraffin or frozen sections of fixed liver, the vacuoles usually appear as optically empty spaces containing no stainable material. In a few cases, however, most of the vacuoles have contained a homogeneous material which stains faintly with ordinary cytoplasmic stains such as eosin. The wall of the vacuole appears smooth, sharp and clear cut. It stains a little darker than the surrounding cytoplasm and presumably represents a separation membrane between the watery contents and the surrounding colloidal cytoplasm.

Although the vacuoles are visible after any of the ordinary methods of fixation, it is best to avoid alcoholic fixatives because these precipitate the cytoplasm in the form of a very open meshwork which may present a rather vacuolated appearance. It is always possible to distinguish between the sharply demarcated true vacuoles and the more indefinite vacuolation of improperly fixed cytoplasm, but in the presence of the latter the search for true vacuoles is more tedious. Formol is not ideal, it precipitates the cytoplasm in coarse lumps and does not harden the tissue adequately for paraffin embedding. In our experience the best cytoplasmic fixative for the liver is a mixture of formol and a metallic salt, as has been used for fixing the Golgi apparatus. Salts of uranium, cobalt and cadmium have been used for this purpose by various workers. We find that copper is equally good, and a mixture of 7 parts 1% copper sulphate and 1 part unneutralized formol has been used for most of this work.

The relationship of the various stainable cytoplasmic components to the vacuoles was investigated by the standard histological techniques. Fat was stained with Sudan IV in frozen sections of formol fixed material by the method of Kay & Whitehead (1935), and also with osmic acid. The vacuoles gave no staining reactions for fat; they were therefore not fat globules (Pl. 1, fig. 12). The cells usually contained a variable number of minute fat droplets; occasionally a few larger ones, about the size of the smallest vacuoles, were present. Once or twice, quite large globules were encountered, but these livers were probably pathological. In paraffin sections, of course, any sizable fat droplets will be represented by empty spaces which will be hardly distinguishable from the watery vacuoles under discussion. It is always essential, therefore, to examine control frozen sections stained with Sudan IV before assuming that the spaces seen in paraffin sections are true watery vacuoles. In practice it was found that the minute fat droplets are too small to appear as visible spaces in paraffin sections, and it was quite uncommon, in the species studied, to encounter any fat spaces at all. Nevertheless, the precaution of fat staining has always been adhered to because of the occasional fatty liver that may be encountered. In the few livers which contained both large fat globules and watery vacuoles, the two were often in close contact. Usually the fat globules were indented by the vacuoles, the fat appearing as a crescent applied to one side of the vacuole; sometimes a thin rim of fat extended round the whole vacuole, which then appeared to lie wholly within the fat globule. Less frequently the watery vacuoles were somewhat indented by adjacent fat globules.

Glycogen was stained by Best's carmine, after Carnoy fixation (Pl. 1, fig. 13); mitochondria by iron haematoxylin or Altmann's acid fuchsin, after Regaud fixation; cytoplasmic ribonucleic acid by toluidin blue after Carnoy fixation (Davidson & Waymouth, 1944). No vacuolar content could be stained by these methods. The glycogen, mitochondria and ribonucleic acid aggregates showed no particular orientation to the vacuoles, nor was there any change in these components as compared with normal cells. When ordinary watery fixatives are employed, the glycogen, after being precipitated in irregular masses, is dissolved out in the water, leaving irregular empty spaces in the cytoplasm. In the case of cells rich in glycogen these 'glycogen spaces' may be quite large, and this normal appearance of glycogen-rich liver cells has sometimes been referred to, incorrectly, as 'vacuolation'. The glycogen spaces are not vacuoles, nor can they be confused with vacuoles, for they are irregular in shape, have no bounding membrane and usually contain a sparse reticulum of cytoplasm. Attempts to stain the Golgi apparatus were unsuccessful. The bile canaliculi were sometimes clearly seen in preparations stained with iron haemotoxylin or Hollande's chlorocarmine, after Carnoy fixation; they were also seen in fresh fragments of liver mounted in plasma. No connexion could be traced between canaliculi and vacuoles; there was nothing to suggest that the vacuoles were distended intracellular bile canaliculi. The 'dark' and 'light' liver cells described by Scharrer (1938) were seen with iron haematoxylin after Bouin fixation, and vacuoles were present equally in both types.

As already mentioned, the vacuoles sometimes contained a homogeneous material which stained lightly with cytoplasmic stains. This 'content' stained a very pale red with eosin, grey with iron haematoxylin, pale bluish grey with Romanowsky stains, and usually blue with the haemalum-aurantia-aniline blue method of Marshall & Trowell (1943). This latter method stains the cytoplasm yellowish-brown and so affords a good differential stain for the vacuolar content. The content is better preserved by formol-copper or formol-bichromate fixatives than by formol alone. It seems unlikely that this content can be anything other than protein, and in appearance and staining reactions it exactly resembles the plasma protein which may be seen in adjacent blood vessels.

The histological conclusion is that the vacuoles consist of a watery fluid which contains a variable amount of protein, possibly plasma protein.

PRELIMINARY OBSERVATIONS

This liver vacuolation was first seen in 1940 during a routine study of histological changes produced in animals by rapid 'decompression'. In these experiments, performed by Eggleton, Elsden, Fegler & Hebb (1945), groups of animals were rapidly decompressed from 1·0 to 0·16 atm. in an atmosphere approximating to pure oxygen. After 2 hr. at the low pressure, by which time some had died, the animals were returned to atmospheric pressure and the survivors killed with coal gas. Autopsies were made at times ranging from 5 min. to 2 hr. after death, and liver samples, examined histologically, showed vacuoles in some, but not all, of these animals. Vacuoles were found both in those which had died as a result of decompression and those which had survived. Vacuoles were produced in this way in the rat, rabbit, guinea-pig, goat and monkey, and these findings have already been reported (Trowell, 1943).

During the war, German workers also discovered these vacuoles. Mueller & Rotter (1942) found them in four airmen who died from the effects of high altitude. Pichotka (1942) found them in guinea-pigs after exposure to an atmosphere of reduced oxygen content at normal pressure. Hesse (1942) found them in humans killed by acute asphyxia (suffocation, drowning). Ladewig (1943) summarized this work and regarded it as proved that the vacuoles were always the result of anoxia occurring during life and believed that vacuolation could be used as a histological criterion for the occurrence of liver anoxia.

In the decompression experiments referred to above, although oxygen was passed through the chamber throughout the experiment, analysis showed that, after decompression, the oxygen partial pressure might be only 80–90 mm. Hg. The animals were therefore subject to some degree of anoxia, and in the light of the German work it seemed that the vacuolation should be attributed to this cause. Certain additional facts were discovered, however, which did not seem to fit in with the anoxia theory. First, since the oxygen tension is presumably least at the centre of the liver lobule, vacuoles might be expected to occur there first; but this was usually not the case. In fifty-three rabbits examined, vacuolation was confined to the centre of the lobules in only three cases; in the others the distribution was midzonal (25), peripheral (18) and throughout the lobule (5). Secondly, in further experiments (carried out variously by J. Fegler, A. W. Missiuro and C. O. Hebb) groups of rabbits were decompressed to various pressures in atmospheres of known oxygen content, and analysis of 200 animals from these experiments showed that the proportion of animals showing vacuoles could be correlated neither with the partial pressure of oxygen to which they had been exposed (range 24–53 mm. Hg) nor with the time for which they had been exposed (range 5–87 min.). Thirdly, vacuoles were found in animals which had been decompressed from 6·0 to

1·0 atm. and had died instantaneously as a result; in this case the question of anoxia seemed to be absolutely excluded.

Histological examination of all the livers obtained from decompression experiments yielded two clues which served as a starting point for further investigation. First, when the livers were classified into three groups on the basis of their glycogen content (histologically assessed), it was found that vacuolation occurred most frequently in the glycogen-poor livers and least frequently in the glycogen-rich livers. This is shown in the case of the rabbit in Table 1. Secondly, it was noticed in all species that the vacuolated livers were always greatly congested with blood.

Table 1. The relation between incidence of vacuolation and liver glycogen content in 200 rabbits dying in decompression experiments

	Total	Vacuolated	% vacuolated
Livers poor in glycogen	58	39	67
Livers moderate in glycogen	90	21	23
Livers rich in glycogen	52	3	6
All livers	200	63	32

It seemed probable, therefore, that anoxia was not the only factor concerned in vacuole formation, and the problem was now investigated by direct experiment.

METHODS

Experiments were performed on adult rats. The rats were starved for 24 hr. before the experiment, in order to avoid high liver-glycogen levels, which, as will be shown later, may prevent vacuolation in conditions under which it would otherwise occur. When anaesthesia was employed, it was necessary to guard against the risk of anoxia resulting from respiratory depression; for this reason 'open ether' was always used. In anaesthetized animals and also with isolated livers it was possible to remove liver samples at intervals during the experiment. The rat's liver has seven accessible lobes, and the distal part of each can be cut off, the stump being previously ligated with string. Using wet string, 1 mm. in diameter, a single twist of a knot tightened gently was sufficient to secure haemostasis without cutting through. After fixation in formol-copper sulphate for 48 hr., each sample was cut in half. Paraffin sections were made from one half and, if these showed vacuoles, then frozen sections stained with Sudan IV were made from the other half to exclude the possibility of fat. Control experiments showed that prolonged ether anaesthesia did not itself produce vacuoles, and that when vacuoles were produced experimentally, their extent and distribution was substantially the same in the different lobes.

RESULTS

(1) *Anoxic death*

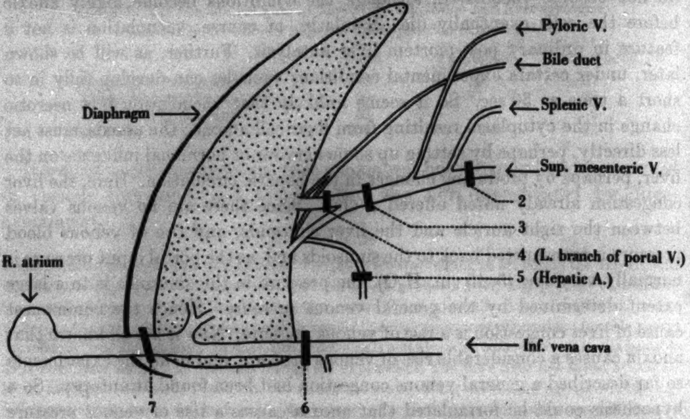
The claim of the German workers that vacuoles are present post-mortem in the livers of animals dying from acute anoxia was confirmed as follows. Twelve rats were killed by placing them, three at a time, in a small sealed chamber containing soda lime. They died of anoxia at times ranging from 20 to 40 min. Liver samples removed post-mortem showed vacuoles in all cases. Six rats killed with coal gas also showed vacuoles post-mortem. In these experiments, as also in the decompression ones, as a result of anoxia the animals over-breathed and became acapnic. But it was easy to show that acapnia is not a necessary factor in vacuole formation because six animals placed in the sealed chamber without soda lime, and therefore dying of asphyxia, also showed vacuoles post-mortem. In all these animals the liver was very congested post-mortem.

In seeking to relate anoxic death to vacuolation, the first and most obvious suggestion is that the vacuolation represents an early degenerative or necrotic change in the cell cytoplasm resulting from a deficiency of metabolic oxygen. But it was found that slices or fragments of liver removed from a living animal, mounted in blood or plasma and kept at 37° C. with exclusion of air, do not become vacuolated, although the conditions become highly anoxic before the cells eventually die. Similarly, of course, vacuolation is not a feature in ordinary post-mortem liver autolysis. Further, as will be shown later, under certain experimental conditions vacuoles can develop fully in so short a time as 30 sec. So it seems unlikely that vacuolation is a necrotic change in the cytoplasm resulting from liver-cell anoxia; the anoxia must act less directly, perhaps by setting up some nervous or hormonal influence on the liver, perhaps by producing changes in the hepatic circulation. Here, the liver congestion already noted offered a clue. Since there are no venous valves between the right auricle and the liver sinusoids, any rise of venous blood pressure is transmitted back to the sinusoids and, as the portal input pressure is normally very low (5–10 cm. H_2O), the pressure in the sinusoids is to a large extent determined by the general venous pressure. Hence the commonest cause of liver congestion is a rise of venous pressure. Now it is well known that anoxia causes a considerable rise of venous pressure, and in all the experiments so far described a general venous congestion had been found at autopsy. So a hypothesis could be formulated that anoxia causes a rise of venous pressure which is transmitted to the liver sinusoids, and the raised hydrostatic pressure in the sinusoids forces fluid from the blood into the liver cells. This possibility was now investigated experimentally.

(2) *Experimental congestion of the liver*

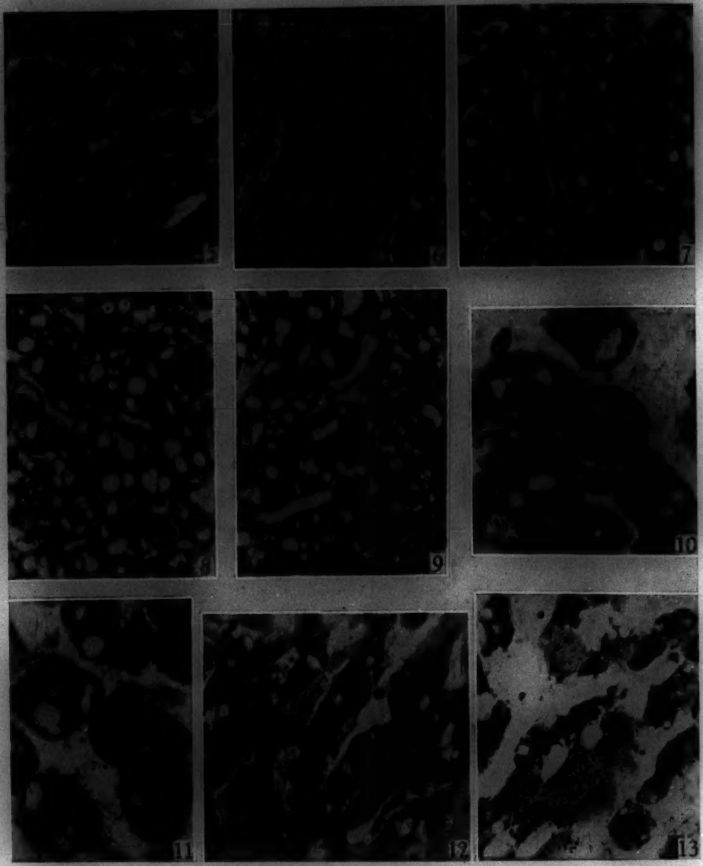
The vascular connexions of the rat's liver are shown semi-diagrammatically in Text-fig. 1, which also indicates by numerals the points at which vessels were ligated or cannulated in the experiments to be described. Under anaesthesia the abdomen was opened and the vena cava ligated at 7. This caused great congestion of the liver, and, of course, blood stasis and anoxia also. Samples removed 10 min. later showed marked vacuolation. In this experiment the blood which entered and distended the liver presumably came from the portal vein and hepatic artery, but it was also possible that some of it came from the vena cava. The pressure in the abdominal vena cava rises considerably, and it is conceivable that it might even come to exceed the portal pressure and so set up a reverse flow through the liver. Such an influx of blood from the vena cava would raise various possibilities, like the entry of large quantities of adrenaline from the suprarenal veins; it was therefore desirable to exclude this factor. In one experiment ligature 6 was first tied, then ligature 7; in another, ligatures 3 and 5 were first tied, then ligature 7. In both cases the liver swelled and congested; in the first case the blood came from the hepatic artery and portal vein, in the second from the vena cava. Both livers became equally vacuolated, so the source of the distending blood is unimportant.

A control experiment to exclude the effects of blood stasis was now performed as follows. Ligatures were loosely placed in positions 6, 5, 3 and 7, and then quickly tied in that order. This completely arrested the liver blood flow, but produced no congestion. Samples removed at intervals up to 2 hr. showed no trace of vacuoles. Comparing this experiment with the previous ones, in both there was complete blood stasis and therefore severe liver anoxia; the only difference was that in the one case the intrasinusoidal blood pressure was approximately zero while in the other it was greater than normal. The experiments described in this section were repeated three times with the same results, and the conclusion was that liver anoxia (or, more strictly, blood stasis) plus raised intrahepatic blood pressure produces vacuoles, whereas liver anoxia with zero intrahepatic pressure does not.



Text-fig. 1. Diagram of the rat's liver showing vascular connexions. The numbers indicate points of ligature or cannulation referred to in the text.

In these experiments the liver was still in nervous connexion, and for a time in hormonal connexion with the rest of the body. It was therefore desirable to carry the isolation of the liver to completion.



Photomicrographs of sections of rats' livers showing watery vacuolation.

Figs. 5–11. Copper-formol fixation, paraffin sections, haematoxylin and eosin.

Fig. 5. Earliest stage of vacuolation, ×285.

Figs. 6, 7. Moderate vacuolation, ×285.

Figs. 8, 9. Extreme vacuolation, × 285.

Figs. 10, 11. Higher magnification to show typical shapes of vacuoles, ×600.

Fig. 12. Frozen section, haematoxylin and Sudan IV, ×285. The vacuoles do not stain.

Fig. 13. Carnoy fixation, paraffin section, haematoxylin and Best's carmine, ×285. Many of the cells are full of glycogen. The vacuoles do not stain.

To face p. 275

(3) *Production of vacuoles in the isolated liver*

Under anaesthesia, a rat was bled out from the femoral vessels, the abdomen was opened and ligatures were tied at 3, 5 and 6. The chest was now opened, and a cannula, inserted into the inferior vena cava through the right auricle, was tied in at 7. The liver, with cannula, was then entirely removed from the body (for details see Trowell, 1942). In the liver there was now complete stasis, anoxia and zero blood pressure; a sample removed at this time showed no vacuoles. The cannula was now attached to a reservoir of heparinized rat's blood, and the liver was distended with a pressure of 20 cm. H_2O. Samples removed 5, 10 and 30 min. later showed great vacuolation. Similar results were obtained if the liver was distended with normal saline and also if saline was perfused through the liver at 20 cm. H_2O pressure so as to remove all the blood. It was thus clear that vacuolation resulted from intrinsic conditions in the liver itself.

By removing samples at successive intervals of time after applying the distending pressure, the development of the vacuoles could be followed histologically. It was found that they first appear as small vacuoles 2–4 μ in diameter; vacuoles less than 2 μ were never seen, so they appear to originate quite abruptly at this size and not by slow growth from minute submicroscopic vacuoles (Pl. 1, fig. 5). They increase in size mainly by individual growth and to some extent by confluence (Pl. 1, figs. 6–9). The region of the lobule in which they first appear is variable; sometimes it is the centre, sometimes the midzone and occasionally the periphery. As the vacuoles increase in size, so the zone of vacuolation extends and ultimately covers the whole lobule. The speed with which the vacuoles develop can be seen from the results listed in Table 2. With a distension pressure of about 20 cm. H_2O the vacuoles are fully developed

Table 2. Vascular distension of the isolated liver (rat). The degree of vacuolation found in samples removed at various times after the application of various distending pressures. Each horizontal line represents a single experiment on one animal

0 = no vacuoles.
+ = small vacuoles in some cells.
+ + = medium-sized vacuoles in the majority of cells.
+ + + = large vacuoles in all cells.

Distending pressure (cm. H_2O)	Time after distension										
	5 sec.	10 sec.	15 sec.	30 sec.	1 min.	3 min.	5 min.	8 min.	12 min.	22 min.	42 min.
Saline, 16	—	—	0	+ +	—	—	—	—	—	+ +	—
Saline, 19	—	—	—	+ +	—	+ +	—	—	+ + +	—	—
Saline, 20	+	—	—	+ + +	—	+ + +	—	+ + +	—	—	—
Saline, 25	0	—	0	+ +	+ +	—	+ + +	—	—	—	—
Saline, 3·5–4·0	—	—	—	—	0	—	—	—	+ +	—	+ + +
Saline, 4·0	—	—	—	—	—	—	—	0	—	+ +	+ +
Blood, 3·5–4·5	—	—	—	—	0	—	—	—	+ +	—	+ + +
Blood, 5·0	—	—	—	—	0	0	+	+	+	—	+ +
Saline, 3·0–3·5	—	—	—	—	0	—	—	0	—	0	—
Blood, 3·0–3·5	—	—	—	—	—	0	—	0	0	—	0

in about 30 sec. and show no further increase with time. When the distension pressure is about 4 cm. H_2O, which is scarcely higher than the normal intrasinusoidal pressure during life, the vacuoles appear only after 5–10 min. and require 40 min. or so for full development; but with a pressure of 3–3·5 cm. H_2O no vacuoles develop at all. The critical level of intrasinusoidal pressure required for vacuole formation was determined more carefully as follows. Starting with 2 cm. H_2O pressure, the pressure was raised in steps of 0·5 up to 5 cm. After each increment, the liver was allowed to stand at that pressure for 20 min., and a sample was then removed to see if vacuolation had occurred at that pressure. In these experiments the animal was bled out, the liver was left in situ and distended by a cannula in the portal vein after ligating at 5, 6 and 7. The pressures were measured from the level of the portal fissure. Three such experiments were performed, and the lowest pressures which produced vacuoles were 4, 4 and 3·5 cm. H_2O respectively. This critical pressure for vacuole formation, 3·5–4 cm. H_2O, corresponds roughly with the normal intrasinusoidal pressure during life. When the distension pressure was 3·5 cm. H_2O, the size of the liver corresponded most closely to its size in the living animal, and McQueen (1929) found the normal intrasinusoidal pressure in the mouse to be about 3 cm. H_2O.

The conclusion is that, in the isolated liver, even with maximal anoxia, vacuoles only develop if the intrasinusoidal pressure is maintained at least at its normal level, and the higher the pressure the more rapidly they develop. It may also be inferred that in the intact animal, vacuolation might occur from liver anoxia even in the absence of congestion, but under these conditions it would take at least 10 min. to develop, whereas if congestion were present it might develop much more quickly.

Perfusion and distension of the liver with histological fixatives (formol, Bouin, Carnoy) at 20 cm. H_2O pressure also produced great vacuolation. This again showed how very rapidly vacuolation can occur, for the vacuoles must have developed before the cells were fixed and fixation must have been very rapid; with Carnoy's fluid the liver became quite hard within a minute. These experiments also proved that the vacuoles develop while the distending pressure is actually applied, for they might have been due to a sudden entry of fluid when the pressure on the cells was released.

(4) *Respective roles of anoxia and congestion*

In this paper the term liver 'congestion' is used to mean any condition in which the intrasinusoidal blood pressure is greater than normal. It has just been shown that, in the presence of the extreme anoxia resulting from complete blood stasis, vacuolation occurs even in the absence of congestion, while if congestion is present as well the process is greatly accelerated. But in the whole animal, during life, anoxia can never be so severe as this and it is questionable if it can ever be severe enough to cause vacuolation in the absence of congestion. Further, in all the experiments so far described anoxia has been present of necessity, and it must be inquired whether congestion alone, in the absence of anoxia, can cause vacuoles. A separation of these two factors, anoxia and congestion, was attempted as follows.

Anoxia without congestion. The simplest way of achieving this is by bleeding. Previous experiments had shown that in the rat, removal of one-third of the blood volume was about the maximum compatible with life. The blood volume was calculated from the body weight (Donaldson, 1924). Under anaesthesia, after opening the abdomen, one-third of the blood volume was removed from the femoral artery. The liver was observed throughout; it was never congested at any stage; after the bleeding it shrank, and remained so. Samples were removed at intervals, and the results of three experiments are shown in Table 3.

Table 3. Anaesthetized rats. The degree of vacuolation found in liver samples removed at various times after haemorrhage

Body weight (g.)	Blood volume (c.c.)	Blood removed (c.c.)	Time after haemorrhage				
			5 min.	15 min.	20 min.	30 min.	45 min.
190	12·0	4·0	—	0	+ +	—	—
130	8·4	2·7	0	+	—	+ +	—
130	8·4	2·5	0	0	—	+	+ +

Vacuolation appeared after 20 min. or so but it never became very marked. The vacuoles remained relatively small, not more than 2 or 3 per cell, and were scattered throughout the lobule. These experiments showed that in the living animal anoxia alone, without congestion, can cause vacuolation, but the vacuolation is rather slight and only develops slowly. Anoxia without congestion was also produced by tying off a part of the blood supply to the liver. Neither ligature of the hepatic artery alone nor ligature of the portal vein alone caused any vacuolation within 30 min. When, however, the vessels were ligated at 2 and 5 (Text-fig. 1), so that the liver received blood only from the pyloric and splenic veins, some vacuolation was present after 30 min. These experiments confirm the view that, in the absence of congestion, only very severe anoxia will cause vacuolation, and even then only to a minor extent.

Congestion without anoxia. The only way to produce liver congestion, without at the same time causing anoxia, is to increase the portal input pressure. But, in so doing, no interruption of the blood flow is allowable, for quite a transitory anoxia might have some effect. Consequently, insertion of cannulae and artificial perfusion are not feasible, and in any case it is not possible to maintain a normal blood flow through the liver by perfusion at normal pressures. So other means had to be sought. In the rat the portal vein, as it approaches the liver, divides into right and left main branches, and these divide further to supply the individual lobes. The right branch supplies two lobes which together comprise about one-third of the liver, while the left branch supplies the remaining five lobes. When the left branch was tied (4, Text-fig. 1) the whole of the portal blood had to pass through the two lobes. These lobes swelled somewhat, and the main portal vein distended to about twice its normal diameter. There was evidently some rise of portal pressure and some congestion of the two lobes, but the congestion was not great. This experiment was performed twice, but no vacuoles were found after 30 min. of such congestion. In a further attempt to produce congestion, 6 c.c. of heparinized rat's blood were slowly injected into the femoral vein of an anaesthetized rat whose calculated blood volume was 14 c.c. This resulted in great distension of the inferior vena cava and moderate distension of the liver. The liver congestion persisted for at least 45 min., and a sample taken at that time showed no vacuoles. In another experiment 5 c.c. of normal saline were injected intravenously into a rat whose blood volume was 11 c.c., and no vacuolation occurred in 35 min. These experiments showed that congestion (to the extent achieved), in the absence of anoxia, does not cause vacuolation. The congestion, however, was less than that which can be achieved by venous obstruction, and it remains possible that more extreme congestion might have had some effect.

(5) *The mechanism of vacuolation*

It is concluded from the foregoing experiments that two factors are necessary for vacuole formation—anoxia and a maintained intrasinusoidal blood pressure. Raised intrasinusoidal pressure (congestion) is an additional augmenting factor which is probably only effective in the presence of anoxia. These are precisely the factors which, in other parts of the body, have been shown to be responsible for the oedema of cardiac failure (Bolton, 1910). This parallelism suggests that, in the case of the liver, anoxia increases the permeability of the sinusoid walls so permitting an increased exudation of tissue fluid. Whereas in other tissues this fluid would accumulate in the tissue spaces, producing oedema, in the liver there is no distensible tissue space between the sinusoid wall and the liver cells, and the fluid therefore passes directly into the liver cells where it accumulates in the form of vacuoles. In other words the vacuoles represent an 'intracellular oedema' of the liver. Evidence for this is as follows.

The vacuoles must represent a watery phase separated by a 'precipitation membrane' from the rest of the cytoplasm which is probably a very viscous colloidal sol. There seem to be three possible ways in which such a watery phase might originate. First, by a physical change in the colloidal state of the cell cytoplasm whereby a watery phase separates out from the colloidal phase. Such changes are known to occur in protoplasm as a result of mechanical injury, pH changes, etc. (Gray, 1931). Secondly, by the loss of some solid constituent, such as glycogen or protein, leaving behind an excess of water which segregates into vacuoles. Thirdly, by an entry of water into the cell from outside. Now in the first two cases there would be no increase in cell volume, whereas in the third case the cell volume would increase proportionally to the vacuolation. When sections from the same liver before and after vacuolation are compared, it is apparent that the vacuolated cells are larger than the original normal cells. In two experiments the average cell size, before and after vacuolation, was measured as follows. In a section of the normal liver, an area comprising about fifty cells and lying about midway between the centre and periphery of a lobule was selected. The outlines of the sinusoids and nuclei were drawn on paper using a camera lucida. The sinusoids were then cut out, the remaining paper was weighed and the weight divided by the number of nuclei gave the average area per cell, measured as mg. of paper. Similar measurements were made on a comparable area in a section of the same liver after vacuolation. The results are shown in Table 4. During vacuolation the average cell area increased by 21 and 31%. Assuming the cells to be cubical the volume increases would be 34 and 51% respectively. There is therefore no doubt that water enters the cells to form the vacuoles.

TABLE 4. The average cell area in sections of normal and vacuolated liver. Areas measured in arbitrary units (mg. paper). Two experiments on rats

	Total area of cells	No. of nuclei	Area per cell	% increase in cell area
Normal	594	50	11·9	
After vacuolation	719	50	14·4	21
Normal	613	47	13·0	
After vacuolation	649	38	17·1	31

The obvious supposition is that anoxia causes water to enter the cells from the blood. But there is an alternative possibility. Water must be passing slowly through the cells all the time, entering from the blood and leaving in the bile and also probably in the lymph. So anoxia might act by arresting this normal outflow of water from the cells. This explanation can be disproved as follows. In the dog, the known normal rates of bile flow and lymph flow from the liver amount, together, to less than 1% of the liver volume per hour; the figure for the rat is not likely to be greatly different. So if the bile and lymph flows were completely arrested the cells could only swell at the rate of 1% of their volume per hour; whereas it has been shown that the actual rate of swelling may be 50% in 30 sec. Further, if this explanation were correct, it should be possible to produce vacuoles by ligating the bile duct and lymphatics. This experiment was performed. Two bull-dog clips were applied to the lesser omentum so as to clamp all the tissue emerging from the portal fissure, with the exception of the portal vein. The bile duct was obviously occluded and subsequent histological sections showed the portal tract lymphatics widely distended, indicating that the lymphatic outflow had been satisfactorily obstructed. Two such experiments were performed, and no vacuoles were found after obstruction for 1 hr. It must be concluded that anoxia acts by increasing the entry of water into the cells from the blood.

The next problem is to determine the force which actually causes water to shift from the blood into the cells. The first possibility is hydrostatic pressure, the difference between the intrasinusoidal blood pressure and the pressure in the cell interior. A second possibility is osmotic pressure; anoxia might cause an increase in the osmotic pressure of the cell contents, and the cells would absorb water. A third possibility is imbibition of water by the cell proteins, but this is unlikely as the water would be bound to the protein and not free to collect in vacuoles.

Now it can be shown that osmotic absorption of water does not lead to vacuolation. If small fragments of liver are mounted in distilled water, the process of osmotic swelling can be followed under the microscope. The nucleus swells at once but later shrinks and becomes pyknotic. The cytoplasm swells only very slowly over a period of several hours. There is a progressive 'thinning' of the cytoplasm which may ultimately come to have a rather frothy appearance, and this appears to be due to swelling of the granules, but discrete vacuoles never appear even after many hours. Further, if osmotic forces are involved, distension of the liver with strongly hypertonic solutions should not cause vacuolation, rather water should be withdrawn from the cells. To test this, an isolated liver was first perfused and then distended at 6 cm. H_2O pressure with 50% sucrose; another was similarly treated with 3% NaCl. In both cases vacuolation occurred just as if normal saline had been used. Samples removed 5 min. after distension showed marked and normal vacuolation, but samples removed at 15 min. and later showed great shrinking of the cells and disappearance of nuclei, though the vacuoles remained. Evidently vacuolation first occurred in the ordinary way, and this was followed by osmotic shrinkage and cell necrosis.

By a process of exclusion it seems that the positive hydrostatic pressure in the sinusoids must be the force which actually causes the water to pass into the cells. The fact that there is a critical level of intrasinusoidal pressure below which vacuolation fails to occur and that raising the pressure accelerates vacuolation strongly supports this conclusion. The question whether, on theoretical grounds, a positive pressure in the sinusoids might be expected to force water into the cells raises very complex mechanical problems concerning the internal architecture of the liver which it is not profitable to discuss here.

The essential cause of vacuolation is not a rise of intrasinusoidal pressure, but a change in the permeability of whatever membranes separate the blood from the liver-cell cytoplasm, a change which allows a more rapid transudation of water even at normal pressures. In the normal liver there must be a net filtration force which tends to drive fluid from the blood into the cells, but actual passage of fluid is opposed by the resistance offered by the intervening membranes, and this resistance is maintained by oxygen. The 'permeability' in question is not that of an inert membrane, it involves forces such as electro-osmosis which are dependent on the vital activity of the cells.

(6) *Vacuolation by distension of the bile canaliculi*

Having shown that, in the anoxic liver, a positive pressure in the sinusoids forces fluid into the cells, it was of interest to see if a positive pressure in the bile canaliculi would do the same. In an isolated liver (bled out) the bile duct was cannulated and distended with saline at a pressure of 25 cm. H_2O. A sample removed after 4 min. showed a few vacuoles just appearing, while a sample at 10 min. showed marked vacuolation. The vacuoles themselves were exactly the same as those seen previously, but their distribution was somewhat different. With bile-duct distension, the vacuoles first appear in the most peripheral cells of the lobules and vacuolation then progresses towards the centre of the lobule in a very regular manner. It seems as if each cell becomes fully vacuolated before the next one is affected at all, and so there is always quite a clear-cut demarcation between the vacuolated zone and the rest of the lobule. After 10 min. the advancing edge of vacuolation had reached about halfway to the centre of the lobule; after 30 min. it had nearly reached the centre. Histologically, the intercellular bile canaliculi were not greatly dilated; evidently they collapse when the pressure is removed, but the vacuoles do not. In another experiment, the pressure was raised in steps, 4·5, 6·5, 10·5, 22 cm. H_2O, at 15 min. intervals. Samples removed at the end of each interval showed that vacuoles did not appear until the pressure was 10·5 cm. H_2O. The critical pressure for vacuolation is therefore higher when the fluid enters from the bile canaliculi than when it enters from the sinusoids, and the speed of vacuolation at comparable pressures is much slower. When the bile duct was similarly distended in a living anaesthetized animal no vacuoles were produced, even after 35 min. distension with a pressure of 25 cm. H_2O. In two experiments the bile duct was tied in the living animal but no vacuoles were produced in 2 hr. It is evident, therefore, that vacuolation from biliary distension, like that from vascular distension, only occurs in the presence of anoxia. The permeability of both sides of the liver-cell is similarly affected by anoxia, but the threshold pressure for water entry differs on the two sides.

(7) *The relation of the liver cells to the blood stream*

The anatomical relation of the liver cells to the blood and lymph is to some extent peculiar, and it will be necessary to define the position before discussing the effects of anoxia on permeability. There has been much discussion as to whether there is a complete endothelial sinusoid wall at all, and, if so, whether there is a space containing tissue fluid between it and the liver cells. The available evidence strongly supports the following view. The sinusoids are lined by a complete endothelial wall. Between the endothelium and the liver cells is a thin film of protein matrix containing scattered reticulin fibres. The terms 'tissue space' and 'tissue fluid' are often wrongly conceived. There are no empty spaces containing free fluid in any normal tissue. The intercellular spaces are filled with a gelatinous protein matrix, and it is the fluid phase of this colloidal matrix which constitutes the tissue fluid. In the liver, therefore, the thin film of protein matrix between the sinusoid wall and the liver cells constitutes a 'tissue space' and contains tissue fluid. There are no lymphatics in this space; the nearest lymphatics are in the portal tracts at the periphery of the lobule, and the tissue fluid has to seep to this region before being picked up. The tissue space in the liver differs markedly from that in other organs in that it cannot be distended by accumulation of tissue fluid. Procedures calculated to cause accumulation of tissue fluid, such as tying the lymphatics and raising the intrasinusoidal pressure (as in the experiments reported in §§ 2, 3 and 5) do not cause any distension of this space; that is, the endothelial wall does not lift off from the liver cells. It seems that the reticulin fibres are firmly adherent to the liver cells on the one side and to the endothelium on the other, thus binding the two together. The sinusoid wall can only be seen as a distinct entity, separate from the liver cells, at certain stages of liver necrosis when the cells shrink and break away from the endothelium, leaving a gap between. When, as a result of increased permeability of the sinusoids, tissue fluid formation is increased, the tissue space cannot distend to accommodate it, so the hydrostatic pressure in the tissue space rises, and tissue fluid is forced into the cells, producing vacuoles. Instead of ordinary intercellular oedema there results what might be termed 'intracellular oedema'.

The total barrier between the blood and the liver-cell cytoplasm comprises the endothelial sinusoid wall plus the liver-cell wall, and the question arises as to which of these is affected by anoxia. Probably both are. Theoretically, if the permeability of the sinusoid wall (only) were increased by anoxia the liver lymph flow should increase, while if the permeability of the liver-cell wall (only) were affected, the lymph flow would not increase. Unfortunately, there is no direct method of measuring normal liver lymph flow. Starling (1894) obtained indirect evidence that in experimental venous congestion the lymph flow from the liver greatly increases, but his experiments involved both congestion and anoxia, and the effect of anoxia alone on the lymph flow from the liver is not known. In the case of capillaries generally, it is known that anoxia increases permeability, and the same might be expected to apply to the liver sinusoids. The experiments in § 6 indicate that anoxia does increase the permeability of the liver-cell wall, at any rate that part of the wall which faces the bile canaliculus. The question must, therefore, be left open, but it seems likely that both the sinusoid wall and the liver-cell wall are affected by anoxia.

It has been shown that, under anoxial conditions, water and also probably plasma protein pass from the blood into the liver cells. The sinusoid wall must, normally, be almost completely permeable to plasma protein, otherwise the osmotic pressure of the protein would oppose the 3–4 cm. H_2O hydrostatic blood pressure, and no filtration would occur. It was of interest to explore further the permeability of the blood/liver-cell barrier. It was evident that sucrose passed freely into the cells, otherwise vacuolation would have been prevented by the high osmotic pressure set up by 50% sucrose. The permeability to haemoglobin was investigated by distending an isolated liver with an 8% solution of haemoglobin (B.D.H.) in normal saline. The liver was first perfused with the solution to remove the blood and then distended at 18 cm. H_2O pressure for 10 min. Histological sections showed vacuoles, most of which contained a homogeneous material which stained to the same depth and colour as the material in the sinusoids. There is no specific staining reaction for haemoglobin (the benzidine-H_2O_2 method fails at this concentration); the nearest approximation is the bright red staining with eosin. The best results were obtained with eosin and pyrrhol blue, which stained the cytoplasm and nuclei dark blue and the haemoglobin in the sinusoids and vacuoles red. Giemsa staining gave the cytoplasm pale purple and the haemoglobin pink. These results indicate that haemoglobin passes freely into the vacuoles.

Colloidal graphite suspensions were tried next. Ordinary Indian ink is unsuitable because the particles aggregate and block the small vessels. Messrs G. Acheson kindly made for me a special graphite suspension in which the majority of the particles were 0·3 μ. This suspension was stable in the presence of blood and also when perfused through the liver. Two isolated livers were perfused with this suspension and then distended at a pressure of 18 cm. H_2O for 15 min. Histological sections showed graphite particles in the sinusoids, lying in a layer against the endothelium but not filling the whole width of the sinusoid. Normal vacuoles were present but no graphite particles were ever seen within them.

When a liver is injected with warm carmine-gelatin by the ordinary method used for the demonstration of blood vessels, vacuoles are produced and the carmine-gelatin enters the vacuoles quite freely. In sections, the vacuoles are seen to contain carmine-gelatin of the same colour and appearance as that in the sinusoids. It will be recalled that Schafer (1902) claimed that carmine-gelatin injection of the liver revealed networks of intracellular blood canaliculi communicating directly with the sinusoids. This led to the belief that the sinusoids had no endothelial wall, the liver cells being in direct contact with the blood. Schafer's work was extended by Herring & Simpson (1906) who, perfusing the portal vein with carmine-gelatin at pressures of 20–40 mm. Hg, with no obstruction to the venous outflow, were able to inject these so-called canaliculi in the rat, rabbit, guinea-pig, cat and dog. They supported Schafer's view that the intracellular carmine represented the injection of a network of blood canaliculi, though they stated that 'the injection frequently has the appearance of rounded or irregularly shaped accumulations, as if in vacuoles of the cytoplasm'. The figures of injected cells given by Herring & Simpson and by Schafer (1902, 1929) do not support their conclusions; they show only independent droplets of carmine, which, though they might sometimes be irregular in shape, were not connected into networks. The carmine-injected livers of rat, rabbit, mouse and guinea-pig which I have examined have shown only round or somewhat irregular droplets of carmine, never networks or any connexion with the sinusoids.

Most histologists have regarded Schafer's canaliculi as an artifact due to rupture of the sinusoid and liver-cell walls by the injection pressure, but Schafer (1929) maintained his original view. It seems more likely that the appearances simply represent carmine-gelatin present in vacuoles such as are here described. Presumably, in anoxia the blood/liver-cell barrier is permeable to carmine-gelatin which enters the cells to form vacuoles, just as saline and plasma have been shown to do. Alternatively, it may be suggested that Schafer was right and that all the vacuoles described in this paper are simply his intracellular canaliculi opened up by distension. Against this interpretation is the fact that 0·3 μ graphite particles do not enter, that even with high distension pressure the vacuoles do not appear at once, and that the vacuoles do not disappear when the pressure is released.

It is concluded that in anoxia the blood/liver-cell barrier is permeable to protein (plasma protein, haemoglobin, gelatin) and to colloidal carmine but not to particles of size 0·3 μ.

(8) *Development of vacuoles post-mortem*

In death from acute asphyxia, both in animals and man, the classical autopsy finding is systemic venous congestion, indicating a considerable rise of venous blood pressure before death.

In rats killed by anoxia or asphyxia it was noted that the liver and great veins were not only distended by blood but distended under some pressure, for, when the inferior vena cava was opened, blood almost spurted out, and the liver quickly deflated. This suggested that, in animals dying of asphyxia or anoxia, the intrasinusoidal pressure did not fall to zero at death, but maintained a positive value for some time post-mortem. Now if the post-mortem pressure were more than 3·5 cm. H_2O, and if it were maintained for 10 min. or longer, then, on the basis of the findings in the isolated liver, vacuolation ought to occur during the post-mortem period, for the liver is then highly anoxic. So it was possible that the vacuoles found post-mortem in animals dead from asphyxia or anoxia had actually developed during the period of post-mortem standing, before the autopsy was made, and not during life. This was investigated as follows. In anaesthetized animals the abdomen was opened, and the animal was then killed by sudden asphyxia. Liver samples were removed at the moment of death (i.e. when the heart stopped) and at intervals thereafter. The liver samples were removed by the method of ligaturing previously described so that no blood escaped from the congested liver. Various methods of asphyxia were used and the results are given in Table 5. These experiments showed that when animals die rapidly from acute asphyxia, no vacuoles form during life, but they develop after about 5 min. post-mortem. If, on the other hand, the animal is bled out as soon as it dies, no vacuoles develop post-mortem. In these experiments the asphyxia only lasted 3–4 min. before the animal died. The effects of a slower asphyxial death were investigated as follows. In a rat under nembutal anaesthesia the trachea was cannulated and connected to a respiration pump, after which the chest was opened. The artificial respiration was now reduced until the animal was asphyxiated and the animal was kept in a condition of maximal asphyxia, just short of death, for 20 min. A liver sample removed at this time showed some vacuoles. The respiration was then stopped, and after 22 min. post-mortem standing the liver showed extreme vacuolation. In another experiment rats were placed, two at a time, in a sealed chamber containing soda lime. When they had almost reached the point of death from anoxia (40–50 min.) they were killed by stunning and at once bled out. It was observed that the heart was still beating. Six animals killed this way all showed some degree of liver vacuolation. These experiments showed that in death from slow progressive anoxia or asphyxia some vacuolation develops before death, but the extreme vacuolation only develops post-mortem.

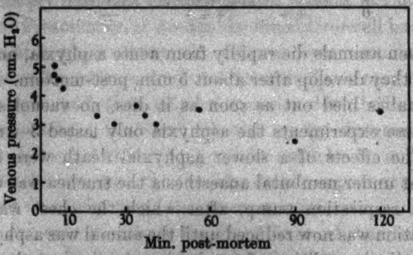
TABLE 5. The degree of vacuolation found in liver samples removed at various times post-mortem in anaesthetized rats killed by sudden asphyxia

Method of killing	Time post-mortem 0	2 min.	4 min.	8 min.	15 min.	20 min.	30 min.
Trachea tied	0	—	0	—	—	+ + +	—
Trachea tied	0	0	—	+	—	—	+ + +
Chest opened	0	0	—	+ +	+ + +	—	+ + +
Coal gas	0	0	+ +	+ + +	—	—	—

It was now evident that the vacuoles found in the original decompression experiments and in the general anoxia experiments (§ 1) were in all probability only formed post-mortem, for the livers were always congested, indicating death from asphyxia or anoxia, and the autopsies were not performed until 5 min. or more after death. The mere fact that the animals died an asphyxial or anoxial death would account for the findings, there is no evidence that decompression or anoxia produced any vacuoles during life, though of course they may have done so. This explanation would account for the lack of correlation between frequency of vacuolation and the degree of duration of anoxia. It would also account for the vacuoles found in the animals which died instantaneously as a result of decompression from 6·0 to 1·0 atm.

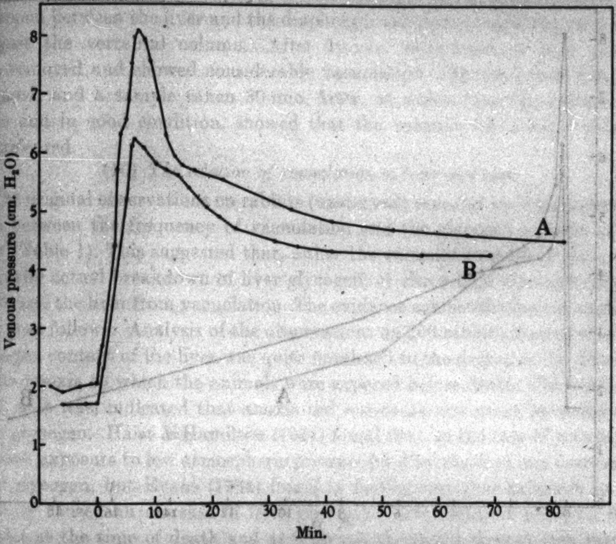
Post-mortem vacuolation was also found consistently in a large series of rats and rabbits (starved) killed by nitrogen or coal gas, and experience suggested the following generalization. In starved animals (rat or rabbit), if at an autopsy performed not sooner than 15 min. from the time of death the liver is found to be congested, then that liver will contain vacuoles.

The development of vacuoles during life can only be proved by finding them in liver samples removed during life, or in samples removed post-mortem from animals which were bled out at the time of death. That liver vacuoles do develop during life, in conditions of severe and prolonged general asphyxia or anoxia, has already been shown in this way.



Text-fig. 2. The venous blood pressure at various times post-mortem in rats killed by anoxia. Each point represents a different animal.

The original argument was that, if, after death, a venous pressure higher than 3·5 cm. H_2O were maintained for at least 10 min., then theoretically vacuolation ought to occur during this period. Having shown that such vacuolation does occur after asphyxial death, it now remains to complete the evidence by showing that the post-mortem venous pressure does fulfil these requirements. The venous pressure was measured by inserting a hypodermic needle, attached to a saline manometer, into the inferior vena cava so that the tip of the needle lay in the intrahepatic part of the vena cava. An ordinary Warburg manometer was used with one limb cut short and connected to the needle. In this way the manometer level could be very rapidly adjusted so as to avoid any entry of saline or withdrawal of blood during equilibration. After insertion, the needle was held firmly in position by a clamp, and at the end of each experiment zero pressure was determined by widely opening the vena cava, leaving the needle undisturbed. In the first experiment twelve rats were killed by anoxia by placing them in a sealed chamber with soda lime. In each animal the venous pressure was measured at a certain time after death, the times differing in the different animals. The results are shown in Text-fig. 2. The next experiments were performed on anaesthetized rats. After opening the abdomen and allowing the wound edges to coagulate, 200 units of heparin were injected into the femoral vein to prevent subsequent clotting in the venous pressure needle. This needle was now inserted and firmly fixed in position. After recording the normal venous pressure the animal was killed,. either by opening the chest or tying the trachea in the neck, and venous pressure readings were made at frequent intervals from the beginning of asphyxia till after death.

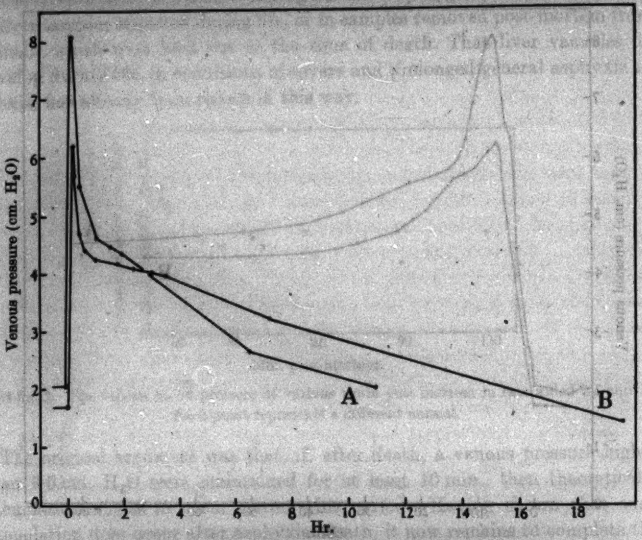


Text-fig. 3. The venous blood pressure before, during and after death in anaesthetized rats killed by sudden asphyxia. At zero time, the trachea tied in Exp. A, the chest opened in Exp. B.

Four experiments were performed and the results of two of them are shown in Text-fig. 3 and on a longer time-scale in Text-fig. 4; the other two gave similar results. It is evident that, after asphyxial death, the intrahepatic venous pressure remained higher than 4 cm. H_2O during the first hour post-mortem and above 3 cm. H_2O for several hours, and so the original prediction is shown to be true.

(9) *Reversibility of vacuolation*

It was discovered that when large (2 cm.^{3}) pieces of vacuolated liver were fixed in formalin, the subsequent sections often showed vacuoles only in the peripheral part of the block and none in the centre. Whereas similar-sized pieces fixed in a rapidly penetrating fixative, such as Carnoy, showed vacuoles throughout. So, if large pieces of liver are fixed in formalin, and the blocks are trimmed down before embedding, vacuolation may be missed. Fortunately this difficulty was discovered early in the work and it is believed that, except perhaps in some of the decompression experiments, no errors have arisen from this source. The simplest interpretation seemed to be that the vacuoles slowly disappear on standing, and that at the centre of a large block they may have disappeared by the time the slowly penetrating formalin reaches and fixes the cells. It was found, however, that pieces of vacuolated liver allowed to stand at room temperature, either protected from air or immersed in saline, for 5 hr. (two experiments) or 24 hr. (two experiments), showed just as many and as large vacuoles as did pieces of the same liver fixed immediately. So it seems that under ordinary conditions the vacuoles do not disappear on long standing in vitro. The explanation of the formalin results is not clear.



Text-fig. 4. The same as Text-fig. 3 but on a longer time-scale.

It was thought that by applying an external pressure to the liver it might be possible to reverse the vacuolation and drive the vacuolar fluid back into the blood. An isolated liver was first vacuolated by distending it with saline, and a sample was removed. The liver plus cannula was then placed at the bottom of a tall glass cylinder with the cannula passing through a cork to the exterior. The cylinder was filled with saline to a height of 20 cm. above the liver. The liver was thus subjected to an external pressure of 20 cm. H_2O, while the vena cava and, theoretically, the sinusoids were in free communication with the outside. After 1 hr. samples showed no reduction of vacuolation as compared with the original sample.

In contrast to this non-reversibility in vitro, it was found that in vivo the vacuoles disappeared fairly quickly when the exciting cause was removed. In an anaesthetized rat the abdomen was opened, and the vena cava was totally obstructed just below the diaphragm. This was done by passing the handle of a teaspoon between the liver and the diaphragm and compressing the vena cava against the vertebral column. After 10 min. obstruction a liver sample was removed and showed considerable vacuolation. The vena cava was now released, and a sample taken 30 min. later, at which time the animal was alive and in good condition, showed that the vacuoles had almost entirely disappeared.

(10) *The relation of vacuolation to liver glycogen*

The original observations on rabbits (unstarved) revealed an inverse correlation between the frequency of vacuolation and the glycogen content of the liver (Table 1). This suggested that, either the vacuoles resulted in some way from the actual breakdown of liver glycogen, or else a high glycogen content protected the liver from vacuolation. The evidence against the former explanation is as follows. Analysis of the observations on 200 rabbits showed that the glycogen content of the liver was quite unrelated to the degree or the duration of the anoxia to which the animals were exposed before death. The evidence, such as it was, indicated that anoxia did not cause any great breakdown of liver glycogen. Haist & Hamilton (1944) found that, in the case of rats fed on glucose, exposure to low atmospheric pressure for 4 hr. resulted in a decrease of liver glycogen, but Evans (1934) found in fasting rats that exposure to low pressure caused an increase of liver glycogen. Liver samples removed from rabbits at the time of death and at intervals thereafter showed (contrary to expectation) that no significant fall in the histological level of liver glycogen occurs during 2 hr. post-mortem standing. Conclusive proof that, in the rat, vacuolation is not due to the breakdown of liver glycogen was obtained as follows. In many of the experiments, already described, in which vacuoles were produced by ligature of the vena cava or by distending the isolated liver, portions of the samples removed were fixed in Carnoy and used for glycogen staining. The sections showed quite clearly that vacuole formation was neither preceded nor accompanied by any change in the histological level of liver glycogen.

Evidence will now be given in favour of the second hypothesis, that a high glycogen content protects the liver from vacuolation. In all the work on the experimental production of vacuoles in rats, starved animals were used, and in them the liver glycogen was always low. Most of these experiments were repeated several times and vacuolation occurred in all cases under the conditions which have been specified. When, however, these same experiments were performed on unstarved animals, vacuolation often failed to occur, and, when it did fail, the liver glycogen was always found to be high. The following experiments on unstarved rats may be cited. Asphyxial death in a closed chamber produced vacuoles in only seven out of sixteen animals, killing by coal gas in five out of ten. Tying the vena cava above the liver produced no vacuoles in one case and a minor degree of vacuolation in three cases. Saline distension of the isolated liver at 6 cm. H_2O pressure gave good vacuolation (one case), slight vacuolation (two cases) and no vacuolation (one case). In three rats the liver glycogen was artificially raised by feeding a high carbohydrate diet for 24 hr. before the experiment. In all three animals distension of the liver with saline at 6 cm. H_2O pressure for 20 min. failed to produce any significant vacuolation, but further distension at 20 cm. pressure for 7 min. did produce considerable vacuolation. Evidently the threshold pressure for vacuolation is higher when the liver is rich in glycogen. From these experiments it was concluded that a high level of liver glycogen may prevent vacuolation in circumstances under which it would otherwise occur.

According to Kater (1933), in the rabbit the liver lobule fills with glycogen from the centre to the periphery, whereas in the rat it fills from the periphery to the centre. So when the glycogen content is small or moderate the glycogen is confined to the centre of the lobule in the rabbit and the periphery in the rat. Now if the concept that a high glycogen content protects against vacuolation applies not only to the whole liver but also to individual cells, then it would be expected that vacuoles should appear first in the outer part of the lobule in the rabbit and in the central part in the rat. In fact, it was found that in both species vacuoles might first appear in any part of the lobule, but it was, nevertheless, true that the centre of the lobule was the least common site for vacuolation in the rabbit and the periphery in the rat. The issue was complicated by the fact that although in most cases the lobular distribution of glycogen followed Kater's description, in some cases it did not. Thus livers were found in which a moderate amount of glycogen was distributed uniformly through the lobule, while in others glycogen-rich and glycogen-free cells were irregularly scattered throughout the lobule; in this latter type it was observed that vacuoles occurred just as frequently in the glycogen-rich as in the glycogen-free cells. Another complicating factor is that, if the vacuoles are due to anoxia, then the oxygen gradient in the lobule should influence their distribution, favouring their occurrence at the centre of the lobule. It is likely that the lobular distribution of vacuoles in any particular case is determined by a variety of factors: the glycogen gradient, the oxygen gradient, and probably other gradients such as intracellular pressure, tissue tension, etc. The most that can be said with regard to glycogen is that vacuoles develop more readily in glycogen-poor livers, that they tend to develop first in that part of the lobule which is poorest in glycogen, but a strict correlation between glycogen content and vacuolation in the case of individual cells cannot be shown. It is known that the liver cells increase in volume considerably when they accumulate glycogen and it has been shown that they increase in volume on vacuolation. So it may be that a cell rich in glycogen is already considerably distended and therefore further distension by vacuolation is more difficult. The explanation may, however, be metabolic rather than mechanical. The liver has some power of anaerobic glycolysis, that is it can obtain energy even in the absence of oxygen by breaking down glycogen to lactic acid. In anoxia, therefore, the vital activity of the cells may come to depend on their glycogen store. There is good reason to believe that the proper permeability of cell membranes is maintained by the energy-yielding metabolism of the cell, so it is possible that even in the absence of oxygen the liver cells can preserve a proper permeability provided that sufficient glycogen is available to maintain glycolysis.

DISCUSSION

Vacuoles such as these have not been described in the normal liver, nor are they ordinarily recognized in human pathology. They have, however, been recorded from time to time in animals under various experimental pathological conditions, but their significance has usually been misinterpreted.

The normal liver. Vacuoles, of a sort, have been described in the normal liver. Noel (1923) described 'neutral red' vacuoles in the normal liver-cell of the mouse. They were minute, few in number, situated close to the nucleus and only visible after supravital staining with neutral red. In the rat and rabbit I have been unable to find any vacuoles staining supravitally with neutral red. Noel also described, as a rapid post-mortem change, vesiculation of the mitochondria leading to the appearance of tiny vacuoles, but these, like the neutral red vacuoles, were very minute and much smaller than the smallest of the vacuoles found in anoxia. Kater (1933) described watery vacuolation associated with the deposition of glycogen in many species, including the rat and rabbit. According to him, all liver cells poor in glycogen contain large watery vacuoles; glycogen is first deposited round these vacuoles and as the glycogen accumulates the vacuoles shrink and disappear, to reappear when the glycogen is withdrawn. I have not seen these appearances, that is, vacuoles and deposition of glycogen around vacuoles, in the normal livers of rat, rabbit, guinea-pig or monkey. Nevertheless, his observation that the vacuolation was greatest in the glycogen-poor cells and least in the glycogen-rich cells is in agreement with what has been found here. Kater does not mention how his animals were killed, but if they were killed by some form of anoxia (e.g. coal gas) then those poor in glycogen would certainly exhibit the post-mortem vacuolation which has been described in this paper, and so it is possible that this is what he was observing. Policard (1934) describes large empty vacuoles present in the liver cells only after meals when the cells are large and distended and says that they are due to absorption of water by the liver during digestive activity. It seems probable that he was simply referring to the glycogen spaces which are normally seen in paraffin sections and which are, of course, a histological artifact. For many years it was believed that the liver-cell contains small secretion vacuoles connected with a fine network of intracellular bile canaliculi (Oppel, 1900), but it is now thought that the appearances were due to staining of parts of the Golgi apparatus and that such vacuoles do not exist (Maximow & Bloom, 1944).

Pathological livers. The watery vacuolation described in this paper seems to have been first observed by Raum (1892). He slowly infused very large volumes of saline into the jugular vein of anaesthetized dogs and killed the animals by tying the trachea about 3 hr. from the start of the infusion. Post-mortem samples of liver showed vacuolation of the cells. He showed that the vacuoles contained neither fat nor glycogen and described them as often ovoid or ellipsoidal rather than spherical and as sometimes indenting the nucleus. He thought they arose from swelling of the fuchsinophil granules (mitochondria), but provided no evidence for this. In one experiment he infused saline for 50 min. and removed a liver sample from the living animal 95 min. later. This sample showed vacuolation. The animal was finally killed after a further 5 hr. and the liver then contained no vacuoles. From this he concluded that the vacuolation was a very transitory phenomenon and he regarded the vacuolation as a 'hydropic infiltration' of the liver cells resulting from hydraemic plethora. He noted that the livers were greatly congested, and there was in addition great circulatory embarrassment, so it would be easy to account for his findings in terms of anoxia and congestion of the liver.

Watery vacuolation has been described in certain types of experimental liver necrosis. Mallory (1901) in a study of liver necrosis in rabbits produced by diphtheria toxin found that watery vacuolation was apparently the first stage in cell necrosis. The vacuoles contained tiny globules or threads of fibrin. It may be mentioned that the anoxic vacuoles described here never contained any fibrin (Weigert staining). Ogilvie (1932) poisoned rabbits with mercuric chloride and after death found that the livers showed congestion, cloudy swelling and sometimes a little necrosis. In three out of sixteen animals the cells showed watery vacuolation, similar to that described here, and this was described as 'hydropic degeneration' of the liver. Drummond & Paton (1904) killed rabbits by injecting very large doses of adrenaline daily until the animal died. Post-mortem they found central degeneration of the liver and in some cases the degenerating cells contained large watery vacuoles. They showed that the vacuoles contained neither fat nor glycogen and believed that they represented an early stage of cell necrosis. All these workers regarded the vacuolation as a degenerative or necrotic change. This may be true, but from the evidence presented in this paper it seems possible, if not probable, that the vacuolation was simply due to venous congestion of the liver produced by the experiments (Mallory and Ogilvie noted that their livers were congested) or perhaps to the fact that the animals ultimately died of anoxia. It is also possible that the various poisons employed damaged the blood/liver-cell barrier, increasing its permeability just as anoxia does. Pappenheimer & Hawthorne (1936) in 31% of 562 routine human autopsies and in twenty-six of thirty-four still-born human foetuses found watery vacuoles in the liver, but the vacuoles each contained a single spherical inclusion body 1–3μ in diameter and sometimes rods and filaments as well. The inclusion bodies were acidophilic and resembled virus inclusions. They were unable to correlate the vacuoles with any disease but noted that they were more numerous in congested livers. They found similar vacuoles with inclusions in normal monkeys, guinea-pigs and ferrets, but not in cats, dogs, rats or rabbits, and they were unable to reach any conclusions as to their significance.

Of greater interest is liver necrosis produced experimentally by prolonged anoxia. Here, one would expect vacuolation to occur but apparently this has not been the general experience. Goldschmidt, Ravdin & Lucké (1937) exposed dogs to an atmosphere of 15% oxygen for 2 hr. and examined the liver 4 days later. They found liver necrosis in some of the animals but did not report vacuolation. Martin, Bunting & Loevenhart (1916) exposed rabbits to 5–9% oxygen for a week and found central necrosis, fatty degeneration and also 'serous imbibition' in some of the cells; probably 'serous imbibition' was their interpretation of watery vacuolation. Cameron & Mayes (1930) tied the hepatic artery in rabbits and examined the livers of animals killed 3, 6 and 12 hr. later. Liver necrosis began at 12 hr. but no vacuolation was reported at any stage. Campbell (1927) exposed rabbits and guinea-pigs to atmospheres low in oxygen, the animals dying after several days. At autopsy the livers were greatly congested and showed fatty degeneration. McMichael (1937) tied portal vein branches in the cat and found intense fatty degeneration of the affected part of the liver 24 hr. later. Neither Campbell nor McMichael mention any control fat staining of their material; doubtless this was done, but, if not, the vacuoles they saw in paraffin sections may have been watery ones. Bolton & Barnard (1931) produced chronic liver congestion in the cat by placing a constricting band round the thoracic inferior vena cava. Animals killed 4 and 6 days later showed central necrosis of the liver; it was also mentioned that 'vacuoles appear' in the cells, but whether they were fatty or otherwise was not stated.

The fact that watery vacuolation has not as a rule been found in these experiments on anoxic liver necrosis may perhaps be explained as follows. It has already been shown that vacuolation in vivo only occurs when anoxia is very severe. It seems likely that in most of these experiments on anoxic necrosis the anoxia was not severe enough to cause vacuolation, but, acting over a period of several days, it was severe enough to cause necrosis. The next point concerns the time-scale of the observations. In most of the experiments the liver was not examined until several days from the beginning of anoxia, whereas the observations described in this paper relate only to the first 1–2 hr. from the onset of anoxia. It has been shown that the vacuolation rapidly disappears when the exciting cause is removed, and it is quite possible that even when the anoxia is maintained the vacuolation may disappear owing to adaptation on the part of the liver. So vacuolation may be only an initial transitory change which the liver rapidly compensates and cures; but there is no direct evidence on this point. Such considerations could explain why watery vacuolation does not (apparently) occur in cases of human chronic venous congestion (nutmeg liver). It is by no means certain, however, that watery vacuolation does not occur in human chronic venous congestion. In such cases the liver cells are greatly vacuolated and although it is generally believed that the vacuoles are fat, it seems desirable that such livers and others showing 'fatty degeneration' should be more carefully investigated with Sudan staining. It may thus be discovered that some of the vacuoles are non-fatty and it may be that watery vacuolation of the liver occurs in human pathology more commonly than has been supposed.

Finally, reference must be made to the production of what appears to be the same watery vacuolation by the feeding of diets deficient in protein. Elman & Heifetz (1941) fed dogs on a low protein diet for 3 weeks and found that the liver cells developed vacuoles which contained neither fat nor glycogen. They believed that the vacuoles were watery and were due to removal of protein from the liver cells. Dean (1942) fed mice on a diet consisting chiefly of sucrose for 2–6 weeks and found similar watery vacuolation of the liver. The vacuoles produced by protein starvation appear, histologically, to be just the same as those produced by anoxia, but it is not at present possible to relate these two sets of findings. It may be that lack of protein in the liver cells or in the blood disturbs the permeability of the blood/liver-cell barrier in the same way that anoxia does.

Addendum. Kritzler (1944) has reported autopsy findings in twenty-seven airmen whose death was attributed to anoxia. In twenty-four cases the liver showed watery vacuolation. In size, shape and numbers the vacuoles were similar to those described in this paper. There was, however, one important difference. In his cases the vacuoles each contained a single eosinophilic inclusion particle apparently the same as those already described in man by Pappenheimer & Hawthorne (1936). Similar vacuoles and inclusions were also found in four out of five fatal cases of carbon monoxide poisoning. In the animal experiments described in this paper such inclusion particles were never seen, though sometimes the vacuoles were filled with a diffuse protein material. It seems likely that this is a species difference in the protein content of the vacuoles and that the fundamental cause of vacuolation is the same. Kritzler also found similar vacuoles in the cardiac muscle fibres, acinar cells of the pancreas, chromaphobe cells of the pituitary, parietal cells of the stomach and sometimes in the thyroid.

SUMMARY

1. A state of watery vacuolation of the liver is described. Discrete vacuoles, 2–12 μ in diameter and approximately spherical, occur in the liver-cell cytoplasm. They are visible in the living cell and contain neither fat nor glycogen. In sections they usually appear as empty spaces, but sometimes they contain a faintly staining material which appears to be protein. This vacuolation has been observed in the rat, rabbit, guinea-pig and monkey.

2. In the starved rat this vacuolation could be regularly produced by any of the following procedures: (i) severe anoxia or asphyxia of the whole animal maintained for 20 min. or more, (ii) venous congestion of the liver produced by tying the vena cava above the liver, (iii) severe haemorrhage, (iv) tying both the hepatic artery and the superior mesenteric vein, (v) distension of the blood vessels of an isolated liver with blood or saline at a pressure greater than 3·5 cm. H_2O—the higher the pressure, the more rapid the vacuolation.

3. In the isolated (anoxic) liver, when the intrasinusoidal pressure was raised to 4 cm. H_2O vacuolation first appeared after about 10 min. and was fully developed at 40 min. When the pressure was 20 cm. H_2O the vacuolation fully developed in 30 sec. The critical pressure required for vacuolation was 3·5–4·0 cm. H_2O.

4. In the starved rat, raised intrasinusoidal blood pressure without anoxia did not cause vacuolation, neither did ligature of the bile duct and liver lymphatics.

5. It was concluded that two factors are necessary for vacuolation, (i) liver anoxia, and (ii) the intrasinusoidal pressure must be maintained at least at its normal value (3–4 cm. H_2O); if it is greater, then vacuolation occurs more rapidly, but, within physiological limits, raised pressure alone does not cause vacuolation.

6. Evidence is presented that the vacuolation is not a degenerative nor necrotic change, that it is due to the entry of fluid into the liver cells from the blood, and that the entry is due to the hydrostatic pressure in the sinusoids and not to osmotic absorption.

7. The theory is advanced that anoxia by altering the 'permeability' of either the sinusoid wall or the liver-cell wall allows the existing hydrostatic pressure in the sinusoids to force fluid from the blood into the cells. The vacuolation could thus be regarded as an 'intracellular oedema' of the liver.

8. In anoxia, plasma protein, haemoglobin and carmine-gelatin, but not colloidal graphite, could enter the vacuoles from the blood stream.

9. The vacuolation was found to be reversible in vivo.

10. In starved rats, dying from sudden (or prolonged) asphyxia, vacuolation developed during the first 10 min. after death provided that the animals were not bled out. After death from asphyxia there is great venous congestion and it was shown that the intrahepatic venous pressure remains higher than 4 cm. H_2O for at least 10 min; so in these circumstances the intrasinusoidal pressure is sufficiently high to produce vacuolation of the post-mortem anoxic liver.

11. In normal (unstarved) animals, the experiments listed in §§ 2, 3 and 10 above produced vacuoles in only 30–50% of animals; vacuolation failing to occur in those with a high liver glycogen. An inverse correlation between vacuolation and the liver-glycogen level was found. It was shown that vacuolation does not result from the breakdown of liver glycogen and it seemed that the glycogen-rich liver is in some way resistant to vacuolation.

12. Distension of the bile canaliculi with saline produced vacuolation in the anoxic liver but not in the normal liver in vivo. The critical pressure for vacuolation in this case was 10·5 cm. H_2O.

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THE RATE AND SITE OF ACETATE METABOLISM IN THE BODY

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The classical β-oxidation theory of fat metabolism postulated the formation of a two-carbon-atom compound as the initial breakdown product of fatty acids, and hence gave to acetic acid a great interest as a probable intermediary metabolite in fat oxidation. The importance of acetic acid in this respect was seriously challenged by the theory suggested by Hurtley (1916) and later elaborated as the multiple-alternate oxidation theory by Jowett & Quastel (1935), according to which the first breakdown product of fatty acids was a four-carbon-atom compound, which was further oxidized without formation of acetic acid. The interest in acetic acid was re-established by the β-oxidation condensation theory of McKay (1943), and strong claims have been made that acetic acid is formed during metabolism (Bloch & Rittenberg, 1944), and that either acetic acid, or some closely related substance such as acetyl phosphate, takes part in the formation of ketone bodies from fatty acids (Weinhouse, Medes & Floyd, 1944). These views make further information desirable about the route, the site and the rate of oxidation of acetic acid in the body.

It is well known that acetate is rapidly metabolized. Schotten (1883), Lamb & Evvard (1919), and Deuel & Milhorat (1928), found that when acetate is administered to animals, only a small amount can be recovered from the urine. Buchanan, Hastings & Nesbett (1943) showed that if acetate containing C^{11} in the carboxyl group be fed to rats, about half of the radioactive carbon appeared in the expired air within 2 hr., indicating the rapid metabolism which occurred. The liver has long been known to be an important site of acetate metabolism, but there is evidence that other tissues may also take part in the process. Toenniessen & Brinkmann (1938) perfused the hindlimb and the liver, and found that in both cases added acetate disappeared from the perfusing fluid. Barcroft, McAnally & Phillipson (1943) found that acetate disappeared from the fluid perfusing the isolated rabbit heart. Evidence on the site of acetate metabolism is also available from experiments with tissue slices. Elliot & Schroeder (1934), Elliot, Benoy & Baker (1935) and Elliot, Greig & Benoy (1937) studied the metabolism of acetate in slices of liver, kidney, testis and brain. Kidney and liver were found to be the most active in causing disappearance of added acetate, while brain and testis did not seem to be effective. The activity of the liver in using acetate was further shown by Leloir & Muñoz (1939), while the metabolism of acetate by the kidney has also been studied by Kleinzeller (1943). The in vitro results suggest that kidney is the most active tissue in acetate metabolism with a Q_{acetate} of about 5, while the Q_{acetate} of the liver is about 2–5. The consumption of acetate by liver and kidney slices has been confirmed by Weinhouse *et al.* (1945) in experiments where acetate containing C^{13} was used. This work also suggested that the routes of oxidation are probably different in these two tissues. In the case of liver the first stage was a condensation to acetoacetic acid, whereas in the case of kidney this did not happen.

The results reported here deal with experiments carried out on the whole animal to determine the rate and site of acetate metabolism, and to investigate how the results obtained on sliced or perfused tissues compare with those obtained under more physiological conditions.

METHODS

Cats, anaesthetized with chloralose, were used throughout the experiments. Solutions of sodium acetate in 2 M concentration, carefully neutralized to pH 7, were injected intravenously in known amounts. Samples of blood were withdrawn at intervals from the femoral vein; the amount of acetate in the blood was estimated, and curves were plotted of the changes in acetate level in the blood. When excretion was to be measured the urine was collected by means of a cannula in the urethra, and the complete emptying of the bladder was controlled by opening the abdomen at the end of the experiment. In order to study the site of acetate metabolism experiments were done in which various organs were removed, and the acetate tolerance curves obtained in such cases were compared with those obtained from the intact animal.

Estimation of acetate. The method used for estimation of acetate was steam distillation with subsequent titration of the distillate. If the procedure of Friedemann (1938) is applied to blood samples it has been shown by McAnally (1944) that there is formation of volatile acid during the distillation process, and that this can be avoided by precipitation of the proteins by magnesium sulphate. In the experiments reported here deproteinization of blood with copper sulphate and sodium tungstate was used, and gave a filtrate which was suitable for distillation. Recovery of acetate added to blood showed that there was no loss of acetate during deproteinization. There are two other possible sources of acid in the distillates besides the acetic acid originally present in the blood. As is well known, steam distillation estimates other substances besides acetic acid, and the substances likely to interfere are the other lower fatty acids and the slightly volatile acids such as lactic, pyruvic, etc. Of the other fatty acids, formic acid has been claimed by Toenniessen & Brinkmann (1938) to be a product of acetic acid metabolism, and might therefore be expected to interfere. To prevent this, all filtrates, before distillation, were subjected to refluxing for 30 min. in presence of mercuric oxide, which causes complete destruction of any formic acid present. The higher fatty acids, propionic, butyric, etc., can be detected in steam distillation by plotting curves for the acid coming over in serial fractions of the distillate. In some preliminary experiments curves of this kind were plotted, and it is seen in Table 1 that the acid obtained in serial fractions of distillate from the blood filtrates corresponded to the amounts obtained from distillation of acetic acid. It must be admitted that the separation of volatile fatty acids by a microdistillation will not have a high degree of accuracy when the total volatile acid present is less than 2 ml. N/100 acid, and greater reliance was placed on the extreme improbability of higher volatile fatty acids being present in amounts which would interfere with the results. The values recorded are therefore total volatile acids in the blood, and the total acid is assumed to be acetic acid. Another possible source of extra acid in the distillates is the mineral acid, usually sulphuric or phosphoric, used for acidification before distillation, and in regard to this the rate of distillation must be considered.

TABLE 1. Percentage of total acid appearing in first 50 and 100 ml. fractions of distillate in steam distillation of pure solutions of volatile fatty acids and of blood filtrates

	Percentage of total acid appearing in fractions of distillate	
Solution distilled (N/100)	First 50 ml. fraction	First 100 ml. fraction
Formic acid	35	60
Acetic acid	44	72
Propionic acid	62	90
Butyric acid	70	96
Blood filtrate	43	70
Blood filtrate	45	75
Blood filtrate	43	72

A convenient rate of distillation was found to be 100 ml. in 6 min. At this rate all the acetic acid came over in the first three 100 ml. fractions of distillate and the mineral acid carried over was small and constant in amount. When a distillation with acetic acid is carried out, the fourth, fifth and subsequent 100 ml. fractions of distillate all give the same small titration value. In the case of distillation of blood filtrate this small titration value will include the slightly volatile acids such as lactic, which may be present, and therefore, if the titration value obtained in the later fractions of distillate is subtracted from the value of each of the earlier fractions, the corrected value for acetic acid will be obtained. The exact procedure used was as follows. Blood was withdrawn from the femoral vein in a heparinized syringe. 2 ml. was pipetted into a tube containing 14 ml. of distilled water. 2 ml. of 7% copper sulphate was added and the tube shaken. 2 ml. of 10% sodium tungstate was added and the tube shaken again. From the clear filtrate obtained 10 ml. was taken for estimation. To the 10 ml. of filtrate, in a boiling tube provided with a ground-glass joint, were added 2 ml. 5% H_2SO_4 and a knife-tip of red oxide of mercury. The tube was attached to a reflux condenser and boiled gently for 30 min. It was then cooled; 7 g. of magnesium sulphate were added, and the tube attached to the distilling apparatus. It was brought to the boil, and then steam distillation commenced at a constant rate of 100 ml./6 min. The distillate was collected in 100 ml. fractions, CO_2-free air was bubbled through for 10 min. and titration carried out with N/100-NaOH. A value of 0·15 ml. was subtracted from the titration value for each 100 ml. fraction, as it was found that this was the blank value obtained in later fractions of distillate. Recovery experiments with acetate added to blood showed that the limit of error in the acetate estimation was about ±7%. Duplicate estimations were always carried out.

Estimation of acetate in urine. In the case of urine where the amount of other acids might be relatively large in proportion to the amount of acetic present, a double distillation was carried out. To the urine was added an equal volume of 10% mercuric chloride and then enough calcium hydroxide suspension to make the solution alkaline. The filtrate was distilled; the distillate was made alkaline and concentrated; then it was acidified and redistilled.

The washing of the distilling apparatus between successive estimations could be greatly accelerated by carrying out between estimations a distillation of a few drops of dilute ammonia solution. This neutralized any acid in the distilling head and condenser. All the ammonia came over very rapidly into the distillate and left the apparatus ready for the next estimation. Care was always taken to see that the distillate was neutral in reaction before starting the next estimation.

Methods of expressing results. The conventional methods of expressing consumption of substrates differ in experiments on sliced or minced tissues and in those where whole animals or whole organs are used. In order to compare the results obtained by different methods the following definitions are given of the terms employed. Throughout this work, concentration of acetate is expressed as millimols (m.mol.) of acetic acid per 100 ml. of blood or body fluid, and consumption of acetate as m.mol./kg. body weight/hr. (1 m.mol. acetic acid = 60 mg.). Q_{acetate} refers to the amount of acetate consumed by a tissue expressed as μl./mg. dry weight of tissue/hr. In expressing the acetate as μl. (0·001 ml.) 1 mol. (60 g.) is taken as occupying a volume of 22·4 l. In making comparisons of results expressed in these different notations the wet weight has been taken as five times the dry weight. It can be calculated that the factor 8·94 will convert the figure for Q_{acetate} to that for m.mol. of acetate/kg. fresh tissue/hr.

In discussing the fate of the injected acetate, certain values have been taken for the volumes of the fluid compartments of the body, as follows: blood volume—70 ml./kg. body weight (Courtice, 1943); extracellular fluid—25% of total body weight; and total body fluid—66% of body weight (Gregerson, 1941).

RESULTS

When acetate is injected into the blood stream in an amount of 20 m.mol./kg. body weight and the concentration of acetate in the blood subsequently followed, an acetate tolerance curve can be plotted as shown in Fig. 1.

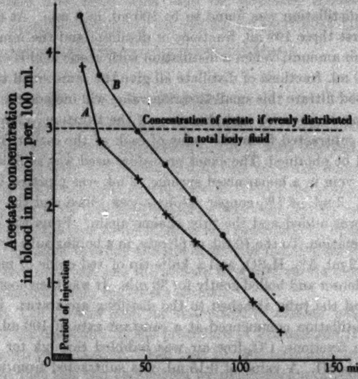

Fig. 1. Acetate tolerance curves in (A) intact and (B) nephrectomized cats. + intact cat. ⊙ nephrectomized cat.

Assuming the values already given for the different fluid compartments of the body, the following calculations can be made. If the injected acetate were evenly distributed in the blood the concentration would be 29 m.mol./100 ml.; if in the extracellular fluid it would be 8 m.mol./100 ml.; and if in the total body fluid about 3 m.mol./100 ml. Hence it is clear from Fig. 1 A that the injected acetate very rapidly disappears from the blood stream. The rapid disappearance might be due to (1) excretion of acetate in the urine, (2) diffusion of acetate into the extracellular and intracellular fluids, or (3) consumption of acetate by the tissues. A certain part of the disappearance is accounted for by excretion in the urine, but only a very small part. In the experiment recorded in Fig. 1 A the total urine secreted during the course of the experiment amounted to 66 ml., and contained 6·75 m.mol. of acetate out of a total of 50 m.mol. injected. Table 2 shows the results of a number of experiments, and gives the amount of acetate appearing in the urine after intravenous injection. The amount lost by this route is seen never to exceed 20% of the total amount given, and was often very much less, so that excretion in the urine contributes relatively little to the disappearance of acetate from the blood stream. This is verified later by experiments in which the disappearance of acetate from the blood stream was studied in nephrectomized animals. The part played by diffusion can be realized by reference to the calculated figures for the hypothetical distribution of acetate in the extracellular and total body fluid. The latter figure is shown as the horizontal line in Fig. 1, and it is seen that the acetate concentration very rapidly falls below that which would correspond to dilution in the total body fluid. It is also evident from the curve shown that there is a very rapid fall in acetate concentration in the blood during the first 30 min. after injection, but that after this the decrease in concentration is represented by a straight line. These observations would suggest that during the first 30 min. after injection the process of diffusion is mainly responsible for the disappearance of acetate from the blood stream, but that after this initial period the disappearance is due to an actual consumption by the tissues.

TABLE 2. Excretion of injected acetate by the kidney

Acetate injected (m.mol.)	Volume of urine (ml.)	Acetate excreted (m.mol.)	Percentage excreted
50·8	50	7·2	14
50·0	40	2·1	4
61·6	124	11·2	18
37·0	17	1·1	3
26·0	43	1·5	6

The acetate tolerance curve, shown in Fig. 1 A, was fairly reproducible when the same dose of acetate, calculated per kg. body weight, was injected into the chloralosed cat. The estimation of acetate is a somewhat laborious process, and as the curve (Fig. 1 A) shows that the rate of fall is represented by a straight line, in most experiments fewer blood samples were taken, and curves were based on fewer points (see Fig. 2). There was clearly a considerable degree of uniformity in these curves, particularly as regards the slope. In a later section will be discussed how the slope can be used to calculate the rate of acetate consumption. At present it is sufficient to point out that the slope is a measure of the rate of acetate consumption.

Role of the liver in acetate metabolism

The influence of the liver on the disappearance of acetate from the blood stream was studied by injection of acetate into animals after the liver had been removed from the circulation. This was done by removing the alimentary tract from the lower end of the oesophagus to the upper end of the rectum, together with ligature of the hepatic artery and portal vein. In these experiments the dose of acetate (20 m.mol./kg.) was calculated from the weight of the animal after completion of the operation.

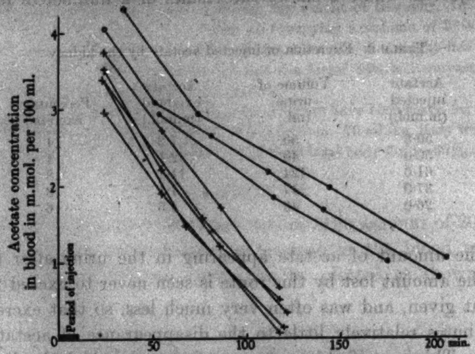

Fig. 2. Acetate tolerance curves in intact and eviscerated cats.
+ Intact animals. ⊙ Eviscerated animals.

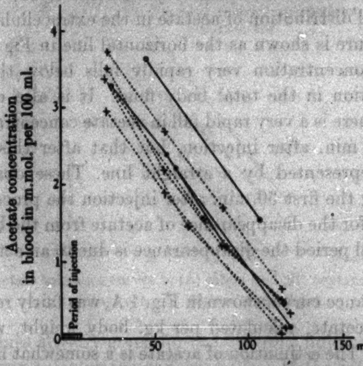

Fig. 3. Acetate tolerance curves in intact cats, and in cats with the hepatic artery left intact, but otherwise eviscerated. Interrupted lines = intact animals. Continuous lines = eviscerated animals.

It is seen from Fig. 2 that there is a very great reduction in the rate of disappearance of acetate after removal of the liver from the circulation. In these experiments the intestine had been removed as well, as it is not possible by any simple procedure to remove the liver without removal of the intestine. The effect of removal of the alimentary tract was controlled by experiments where the intestine was removed, the portal vein tied, but the hepatic artery left intact, and hence the liver still maintained in the circulation, at least in as far as it was supplied by the hepatic artery but not by the portal vein. It is evident from Fig. 3 that the effect of removal of the liver and intestine in the first set of experiments was due solely to removal of the liver.

Role of the kidney in acetate metabolism

The experiments of Elliot *et al.* (1934, 1935 and 1937) and of Kleinzeller (1943) suggest that the kidney plays a predominant part in the metabolism of acetate. This function of the kidney was investigated in a series of experiments in which acetate tolerance curves were made in nephrectomized animals, and compared with those obtained in intact animals. In Fig. 1 two curves are shown, one from an intact animal and one from a nephrectomized animal, in each case with blood samples taken at 15 min. intervals. In both cases the disappearance of acetate is represented by a straight line after the first 30 min. The curve for the nephrectomized animal lies at a slightly higher level, which is probably due to excretion of acetate by the kidney in the intact animal during the initial stages of the experiment, while the blood concentration is still high. The slope of the curve does not indicate any faster rate of metabolism in the normal animal than in the nephrectomized, and indeed indicates a slightly slower rate. This would indicate that the kidney does not

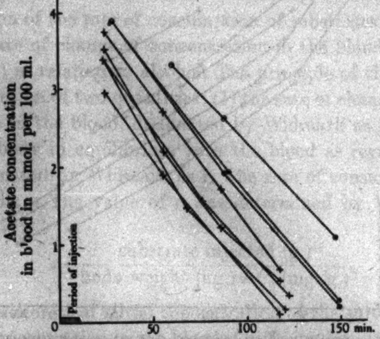

Fig. 4. Acetate tolerance curves in intact and nephrectomized cats.
+ Intact animals. ⊙ Nephrectomized animals.

take any special part in acetate metabolism. Fig. 4 shows the results of a series of experiments, in which fewer points on each curve were obtained. It is evident from these also that any increase in rate of acetate disappearance from the blood in the normal animals over the rate in the nephrectomized animals is very small. When the curves are compared with those in Fig. 2 obtained with eviscerated animals it is seen that the kidneys play a very much smaller part than the liver in acetate removal. The weight of the two kidneys in the cat is about one-third that of the liver, so that, considered per unit weight of tissue, the kidneys are still much less active than the liver in acetate removal. If the activity of the kidney tissue in acetate removal was twice as great as the liver tissue, as suggested by the in vitro results, then removal of the kidneys should have an appreciable effect on the slope of the acetate tolerance curve.

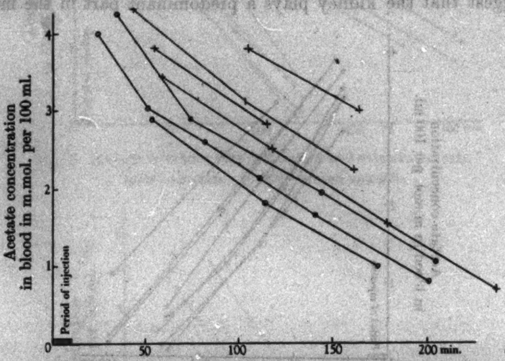

Fig. 5. Acetate tolerance curves in eviscerated cats and in cats eviscerated and nephrectomized. ⊙ Eviscerated animals. + Eviscerated and nephrectomized.

In order to investigate the problem further, a series of experiments was done in which the rate of disappearance of acetate was reduced by removal of the liver, and comparisons made with nephrectomized animals and animals with kidneys intact. It was hoped that by this means small changes in the rate of disappearance of acetate due to removal of the kidney might become more obvious, or that the kidney might show a greater capacity to deal with acetate if the removal of acetate by the liver was not going on at the same time. These experiments on eviscerated animals and on animals eviscerated and nephrectomized completely confirmed the results in animals with liver circulation intact (Fig. 5). The slope of the curves for acetate disappearance was not affected by removal of the kidneys except for the initial stage where the fall in concentration of acetate was more rapid in the intact animal.

Metabolism of acetate by blood

The blood was the only other tissue specifically examined. Acetate was added to blood and incubated in Warburg flasks at 37° C. with continuous shaking for several hours. The results are shown in Table 3. It is evident that the blood does not take any part in the breakdown of acetate.

TABLE 3. Effect of blood on acetate in vitro

Species	Period of incubation at 37° C. (hr.)	Concentration of acetate (m.mol./100 ml.) Initial	Final
Cat	2	2·2	2·1
Cat	3	2·2	2·2
Cat	2	2·9	2·9
Cat	2	2·9	2·8
Rabbit	3	2·0	2·0

The rate of consumption of acetate by the body

The calculation of the rate of consumption of substrates from the measurement of their rate of change of concentration in the blood has been used by Widmark (1932) in relation to alcohol. The principle of the method depends on the determination of two quantities: (1) the rate of change of concentration of the substrate in the blood, designated by Widmark as β, and (2) the proportion of the body in equilibrium with the blood as regards the particular substrate, designated by Widmark as r. The rate of consumption of the substrate is then $\beta \times r$. The value of r was determined by Widmark from the following formula:

$$r = \frac{\text{substrate injected (g.)}}{\text{body weight (kg.)} \times C_0 \text{ (mg./g.)}},$$

where C_0 is the theoretical initial concentration of the substrate in the blood, i.e. the initial concentration in the blood if diffusion into the tissues had been instantaneous. This method was also used by Eggleton (1940*a*) in relation to alcohol consumption, and in this case the calculation was verified by determining the rate of injection of alcohol which would maintain a constant level in the blood. For the calculation of the rate of consumption of acetate the formula has been modified as follows:

$$r = \frac{\text{acetate injected (m.mol./kg.)} \times 100}{C_0 \text{ (m.mol./100 ml.)}},$$

where r expresses the volume of body fluid in ml./kg. body weight, which is in equilibrium with the blood. The rate of consumption is then $\beta \times r/100$.

The value of β is determined from the acetate tolerance curves, and will be expressed as m.mol. acetate per 100 ml. blood/hr. The value of C_0 has to be determined from backward extrapolation of the straight part of the acetate tolerance curve. The method of doing this is shown in Fig. 6 which is modified from a diagram illustrating calculation of alcohol consumption by Eggleton. From this it is seen that the backward extrapolation of the straight part of

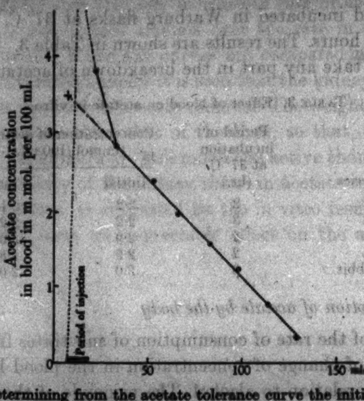

Fig. 6. Method of determining from the acetate tolerance curve the initial hypothetical concentration of acetate in the blood by backward extrapolation. ⊙ Values determined experimentally; ⊙—⊙ Acetate tolerance curve. - - - - Probable course of initial part of curve. ·—· Backward extrapolation of straight part of tolerance curve. + hypothetical initial concentration of acetate in blood.

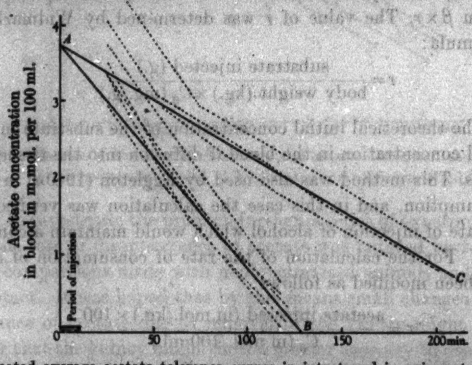

Fig. 7. Estimated average acetate tolerance curves in intact and in eviscerated animals. The actual curves obtained are shown in interrupted lines. The line *AB* represents the average curve for the intact animals. The line *AC* represents the average curve for the eviscerated animals. In both cases the point *A* represents the hypothetical initial level of acetate concentration in the blood.

the curve intersects the ordinate at zero time at about 3·5. The dose of acetate injected was 20 m.mol./kg. body weight, and therefore the value of *r* will be 570. The rate of fall of concentration of acetate in the blood can be worked out from the curve as 1·55 m.mol./100 ml. blood/hr., and therefore the rate of consumption is 8·8 m.mol./kg./hr. In Fig. 7 is shown a number of curves obtained from normal and eviscerated animals, and two lines, *AB* and *AC*, drawn to represent the average curves in the two cases. According to these results the value of C_0 is 3·7 and of r therefore 540. The slope of the line *AB* corresponds to a rate of disappearance of acetate of 1·72 m.mol./100 ml./hr., and therefore the consumption of acetate is 9·3 m.mol./kg./hr.

Estimation of the rate of consumption by constant infusion

The rate of acetate consumption was also investigated by administering to animals an infusion of acetate solution at a rate so as to maintain the concentration of acetate in the blood at a constant level. This was done in the following way. The animal was nephrectomized, so as to eliminate any possibility of excretion of acetate in the urine. It has already been shown that nephrectomy does not appreciably alter the rate of acetate metabolism. An initial dose of acetate was given of such size as to bring the blood concentration to a suitable level after 1 hr., in which time it can be seen from Fig. 1 that the equilibrium due to diffusion of acetate into the tissues is complete. A constant intravenous infusion was now commenced, and blood samples were taken at the beginning and end of a 40 min. period. At the end of the period the rate of infusion was altered and the process repeated. Table 4 shows the results of two experiments of this kind. The consumption of acetate is somewhat lower than that arrived at by calculation from the tolerance curves, and is seen to be about 6–7 m.mol./kg./hr. in comparison with a value of about 9 arrived at by calculation. For alcohol Eggleton found a closer correlation (within 8%) of the results obtained by calculation and by constant infusion method. Why there should be a greater discrepancy in the case of acetate is not clear, but it would seem safe to take the figures obtained by the constant infusion method as more likely to be correct.

TABLE 4. Estimation of acetate consumption in the nephrectomized cat by constant infusion method

Weight of cat (kg.)	Rate of infusion (m.mol. acetate/hr.)	Acetate concentration in blood (m.mol./100 ml.)		Estimated rate of consumption (m.mol. acetate/hr.)	
		At beginning of period	At end of period	Total	Per kg. body weight
2·12	10·0	1·09	0·77	Between 11·4 and 14·9	Between 5·4 and 7·0
	11·4	0·77	0·61		
	14·9	0·61	0·79		
	19·8	0·79	1·26		
3·49	20·6	0·76	0·51	Between 20·6 and 23·5	Between 5·9 and 6·7
	23·5	0·51	0·58		
	26·3	0·58	0·75		
	30·4	0·75	1·05		
	41·6	1·05	1·73		

The rate of acetate metabolism by the liver

In Fig. 7 the line *AB* gives the average rate of acetate metabolism in the intact animal and *AC* the average rate in the eviscerated animal. The slopes of these curves represent a fall in acetate concentration of 1·72 and 0·85 m.mol./100 ml./hr. The liver is thus seen to be responsible for about half the acetate metabolism in the animal. This figure was tested by the constant infusion method with animals which had been both eviscerated and nephrectomized. The results are shown in Table 5. According to these the consumption of acetate in eviscerated and nephrectomized animals is about 3·5 m.mol. acetate/kg./hr., or just over half the value for the entire animal. This agrees well with the relative rates calculated from the slopes of the curves.

TABLE 5. Estimation of acetate consumption in the nephrectomized and eviscerated cat by constant infusion method

		Acetate concentration in blood (m.mol./100 ml.)		Estimated rate of consumption (m.mol. acetate/hr.)	
Weight of cat (kg.)	Rate of infusion (m.mol. acetate/hr.)	At beginning of period	At end of period	Total	Per kg. body weight
2·23	6·1	1·06	0·65	8·4	3·7
	7·5	0·65	0·50		
	8·4	0·50	0·50		
2·44	6·3	1·25	0·85	About 8·7	3·50
	7·4	0·85	0·63		
	8·7	0·63	0·65		
	10·0	0·65	0·93		

From these results the consumption of acetate by the liver would appear to be about 3 m.mol./kg. body weight/hr. The size of the liver in the cat is somewhat variable. Stadie, Zapp & Lukèns (1940) gave a value of 26·3 ± 1·5 g./kg. Eggleton (1940*b*) found in two series of experiments the values 2·3 ± 0·1 g. and 3·4 ± 0·1 g./100 g. In the cats used in the present series the liver weights varied from 23 to 27 g./kg., but this does not include any of the animals used in evisceration experiments, as it was found that after the liver had been shut off from the circulation for some time the average weight was much greater. Even if the value 30 g./kg. is taken for liver weight, and the value 3 m.mol./kg. for acetate consumption, the consumption of acetate by liver tissue amounts to 100 m.mol./kg. or to a Q_{acetate} of 11. Although this calculation has been made on rather conservative estimates, it exceeds by 2–5 times the values obtained by Elliot *et al.* (1937), Kleinzeller (1943) and by Leloir & Muñoz (1939) for liver slices.

Certain criticisms might be made of the experiments on which are based the calculated rates of acetate metabolism by the liver. It is known that ligature of the portal vein and hepatic artery does not completely exclude the liver from the circulation. If the liver in the eviscerated animal is still in contact with the blood stream it implies that the rate of acetate metabolism may be even greater than that suggested by the figures given here. On the other hand, the fact that evisceration reduces acetate metabolism only by about one-half suggests that the extrahepatic tissues play a large part in acetate metabolism in the eviscerated animal, as it is improbable that ligature of the portal vein and hepatic artery only halves the rate of removal of acetate from the blood stream by the liver. In the experiments where the hepatic artery circulation was maintained after ligature of the portal vein it might be argued that the liver was not provided with an adequate blood supply. Since actually the rate of acetate consumption in these animals was as great as in the intact animal, this cannot invalidate the conclusion that the fall in acetate consumption in the eviscerated animal must be attributed to removal of the liver and not to removal of the intestine. Another possibility which must be considered is that the intestine in the intact animal may act as a source of acetate, as was shown by Barcroft, McAnally & Phillipson (1944). In their experiments the amount of acetate absorbed into the blood stream from the intestine was very small, except in the case of the caecum and part of the stomach of the herbivorous animal; it is not therefore likely to be significant in the cat.

Acetic acid as a source of energy

The oxidation of 1 g. acetic acid liberates 3·75 kg.cal. It has been shown that the rate of acetate metabolism in the anaesthetized intact cat amounts to about 7 m.mol./kg./hr. This is equivalent to a heat production of about 1·5 kg.cal./kg./hr. What is more significant perhaps is the total heat production from the oxidation of the fat from which this acetic acid might be derived. Assuming that the fatty acid chain is broken down into two-carbon units which form acetic acid, then the amount of acetic acid from one molecule of a mixed fat (oleopalmitostearin) is 26 mol. The amount of fat which would give rise to 1 g. of acetic acid would liberate 5·06 kg.cal., and hence the rate of acetic acid metabolism which has been found in these experiments would correspond to a heat production from fat of about 2 kg.cal./kg./hr. As the oxygen consumption was not measured it is not known what fraction this represents of the total metabolism, but it must be a considerable one since the basal metabolism of the *unanaesthetized* cat is less than 3·0 kg.cal./kg./hr. (Ring & Hampel, 1932).

By using the figures of Best, Dale, Hoet & Marks (1926) it is possible to compare the rate of energy production from acetate and glucose. In an eviscerated decapitated cat weighing 3·2 kg. there was an oxidation of 1·045 g. glucose in 50 min. This is equivalent to a rate of consumption of 0·39 g./kg./hr., and to a calorie production of 1·6 kg.cal./kg./hr. This is about the same rate of energy production as oxidation of acetate could cause in the whole animal. Of interest in relation to fat metabolism is the value estimated by Stadie (1942) for the fat metabolism of the diabetic cat. On the basis of ketone-body production by the liver of the diabetic cat he calculated that this could account for a metabolism of 2·3 g. of fat/kg. body weight/day. If this amount of fat were converted to acetic acid as a first stage in ketone-body production it would require a rate of acetate metabolism by the liver of about 3 m.mol./kg. body weight/hr., and this is in fact the value for acetate metabolism which has been obtained in the present series of experiments. All these calculations suggest that the body can metabolize acetate sufficiently rapidly to enable it to act as a possible metabolite in fat oxidation.

DISCUSSION

Whether acetic acid is or is not an intermediary product of fat metabolism must still be considered as undecided. The two-carbon atom unit which is probably formed in the liver has not yet been proved to be acetic acid, but on the other hand the fact that Stadie (1942) failed to find acetic acid in liver slices does not prove that it is not formed, since the isolation of acetic acid from tissues presents certain difficulties (Lehninger, 1942). The results obtained in the present experiments show that acetic acid is metabolized by the liver at a much more rapid rate than had previously been demonstrated, and from this point of view strengthen its claims to be considered as an intermediary product of metabolism. They also suggest other possibilities about the fate of acetic acid if it is formed. Most of it will presumably be metabolized in the liver. Whether any enters the blood stream will depend on the relative rates of its formation and removal by the liver. Evidence based on the rates of reactions in vitro alone cannot decide this point.

The amounts of volatile fatty acid in the blood are small but appreciable. McClendon (1944) found a value of 0·03 m.mol./100 ml. blood. This low figure is evidence against the formation of acetate by the liver and its utilization by the peripheral tissues in large amounts, but in view of the considerable capacity of the extra-hepatic tissues to deal with acetate, and following Toenniessen & Brinkmann (1938) the suggestion is put forward that a part of the acetate formed in the liver may be carried by the blood stream and oxidized in the tissues. Acetic acid might represent a 'quick fuel' derivable from fat in the sense employed by McKay (1943). This suggestion is in no way incompatible with the view that most of the fat oxidation takes place by way of ketone body formation in the liver, a view which is supported by the very great capacity of the liver to metabolize acetic acid. The fact that acetic acid is easily metabolized by the extra-hepatic tissues suggests that it might also be an intermediary metabolite in the direct oxidation of fat by the tissues.

The in vitro experiments have assigned to the kidney an important role in acetate metabolism, but this view has not received support from the present work. It must be admitted, however, that while the role of the kidney in acetate metabolism is small in comparison with that of the liver, the rate of acetate metabolism by the liver has been found to be very much higher than would be concluded from the in vitro experiments. This fact, together with the small size of the kidneys in comparison with the other extra-hepatic tissues makes one hesitate to draw conclusions about the absolute rate of acetate metabolism by the kidney. Another possible explanation of the failure to find a more active participation by the kidney in acetate metabolism may be the presence of other substrates which are preferred to acetate. In experiments with kidney slices, Krebs (1935) found that when two oxidizable substrates were present there was no summation of oxygen consumption, but a competition for the available oxygen and displacement of one substrate by the other. Another possible factor is the species difference. The in vitro experiments have been done with rabbit and rat kidney, while in the present experiments only the cat was used. It would be valuable to study the in vitro activity of the cat's kidney with regard to acetate.

Since the extra-hepatic consumption of acetate is not accounted for by the kidney, it is difficult to avoid the conclusion that muscle probably takes a part in acetate metabolism. This is suggested by the experiments of Toenniessen & Brinkmann, and of Barcroft *et al.*, although Krebs (1943) has considered that the part taken by the muscles in acetate metabolism is small. The role of muscle tissue in acetate metabolism requires further investigation, but the present experiments make it clear that other tissues apart from liver and kidney considered as a whole and not per unit weight can metabolize acetate at a rate about as fast as the liver metabolizes it. This is supported by a brief report by Dye & Masters (1944), who found that in nephrectomized dogs the extra-hepatic tissues could account for up to 70% of the acetate metabolized.

The results reported also provide an opportunity to compare the absolute rate of metabolic activity in in vitro and in vivo experiments. The Q_{acetate} of liver tissue usually amounts to about 2 in the in vitro experiments, while in the present case it amounts to about 11. It is unusual to find the activity of tissues in vivo so much greater than their activity in vitro. For the oxygen consumption and pyruvate metabolism of heart muscle Smyth (1940) found that the in vitro rates were about as great as the maximum in vivo rates, when the heart was working at full capacity.

It is of interest to compare the rate of metabolism of acetate with that found for alcohol by Eggleton (1940*a*) in similar experiments on the cat. The rate of metabolism of alcohol was about 175 mg./kg. body weight/hr., which is equivalent to 3·8 m.mol./kg./hr. This is much less than the rate at which acetate can be metabolized by the body, but on the other hand rather greater than the rate at which acetate can be metabolized by the liver alone. This would explain why Lundsgaard (1937) found evidence of accumulation of acetate when alcohol was being metabolized by the perfused liver, while

Himwich, Nahum, Rakieten, Fazikas, du Bois & Gildea (1933) could find no evidence of accumulation of acetate after administration of alcohol to the whole animal.

SUMMARY

1. Acetate tolerance curves have been made in intact, nephrectomized and eviscerated cats in response to intravenous injection of acetate solutions.

2. Excretion of acetate in the urine accounts for only a small part of the acetate disappearing from the blood stream.

3. The liver accounts for about half the acetate used, and the activity of liver tissue in metabolizing acetate is much greater than that suggested by in vitro experiments.

4. The kidney does not take any more prominent part than the other extrahepatic tissues in acetate metabolism.

5. The nephrectomized and eviscerated animal is capable of metabolizing acetate at about half the rate of the intact animal.

6. The relation of these findings to the possible role of acetate as an intermediary metabolite in fat metabolism is discussed.

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THE COLOUR SENSITIVITY OF THE RETINA WITHIN THE CENTRAL FOVEA OF MAN

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In 1894 König noticed that if the size of a coloured object was sufficiently reduced and that if it was observed by the eye so that its image fell upon the central fovea, all colours could be matched by a mixture of a red and a blue stimulus only, i.e. that vision under these conditions was dichromatic. König also discovered that the ability to differentiate between wavelengths in the blue-green region of the spectrum was impaired and that the type of vision was similar to that found in the tritanopic form of colour blindness.

Recently, Willmer (1944), and Willmer & Wright (1945) have confirmed and extended König's observations. Luminosity, dichromatic coefficient and hue-discrimination curves were measured and spectral mixture curves calculated for a small circular field whose diameter subtended an angle of 20′ at the eye. The image of this field lay on the retina at the fixation point within the central fovea, and vision for this field was found to be dichromatic and tritanopic. Fixation of the object could only be maintained momentarily and if fixation was imperfect the dichromatic match was at once disturbed. Hartridge (1945*a*, *b*), using as his test for dichromatic vision the fact that yellow is seen as white, has also investigated the sensitivity of the fovea with a series of small yellow-painted test objects, and compared this sensitivity with that of the surrounding retina. He concluded that if an object were sufficiently small, it would exhibit the characteristics of dichromatic vision not only at the fovea but wherever its image lay on the retina. It is clear that in his view, retinal position is of no importance, and that it is the reduction of size alone which initiates the effects described by König. He inferred from his experiments that there was no significant difference between vision at the foveal centre and that within a much larger retinal area.

The present series of experiments were designed to record the characteristics of vision for different points within the central fovea. The luminosity, coefficient and hue-discrimination curves were again recorded for a 15′ test field when viewed at 20′ and 40′ from the foveal centre and the results have been compared with the data for central fixation. For greater displacements than this, a larger test field must be used to compensate for the reduced visual acuity and no measurements of this type are reported here.

METHOD

The observing fields were of two types. In type A, Fig. 1, the test and comparison beams formed the two halves of a small circular field, the diameter of which subtended an angle of 15′ at the eye. Displacement of the field was effected by the provision of two points of light to one side of the circular field so that, if the appropriate point was fixated, the centre of the circular field would then be displaced from the fixation point through an angle of either 20′ or 40′. In type B, Fig. 1, the size of the two halves of the field was the same as that in type A, but the lower semi-circular patch *a* could be compared with any one of the three upper patches *a′*, *b′* and *c′*, where the angular displacements of *b′* and *c′* from *a′* were respectively 20′ and 40′. With an arrangement of this type it

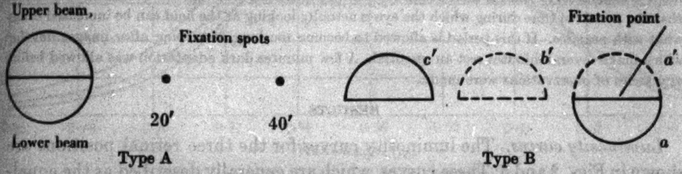

Fig. 1. The two types of observation made. This diagram of the test field will subtend an angle of 15′ if it is held at a distance of 12 ft. from the eye.

was possible, by fixating *a*, to obtain a comparison of the relative sensitivities of the central, 20′ and 40′ areas. All observations were made with the right eye and the displacements from the foveal centre brought the test fields into the nasal half of the visual field (temporal side of the retina).

The measurements were made with the colorimeter developed by one of us (Wright, 1927, 1939) in which a wide variety of spectral lights, with independent intensity control, may be applied to each of the two halves of the circular field. Correct alinement and fixing of the head relative to the exit pupil of the instrument were obtained by biting upon a suitably placed dental impression mouthpiece. A further addition to the optical system was the introduction, immediately behind the exit pupil of the colorimeter, of a small lens with sufficient chromatic over-correction to correct for the chromatic aberration of the eye. The lens was designed to have approximately zero power for yellow light but to give +3·0 D. chromatic over-correction between the wave-lengths 0·70 μ. and 0·40 μ. The specification of the lens was as follows:

Hard crown biconvex component, $n = 1{\cdot}5190$, $v = 60{\cdot}4$.
Radius of curvature of each surface = 0·33 in.
Thickness = 0·10 in.
Diameter = 0·33 in.

Dense flint biconcave component, $n = 1{\cdot}6226$, $v = 36{\cdot}0$.
Radii of curvature of surfaces = 0·33 in. and 0·419 in.
Thickness = 0·05 in.
Diameter = 0·33 in.

Crown and flint components cemented together.

The fitting of this lens greatly increased the certainty and consistency with which observations could be made in the blue and violet region of the spectrum, since without the lens the area of retinal stimulation must vary according to the wave-length of the light and the focusing of the observer's eye. The small circular field and fixation points were produced by placing a metal plate in which suitable holes had been drilled, between the photometer prism and the eyepiece of the apparatus. The fixation points in type A experiments were thus illuminated by light from the colorimeter prism and were of the same colour and brightness (but lower intensity) as the circular field itself. An exit pupil 2 mm. in diameter was used and the field brightness in all experiments was approximately 50 photons.

Technique. In making these measurements some experience was necessary before one could be certain that the correct retinal area was being used. To hold the eye rigidly in the correct position for more than a few seconds at a time was quite impossible. Measurements were therefore made in the following way. A 'bite' was taken and, by looking at the field, the head position was checked. Minor inaccuracies of head position do occur even with the bite method of fixation and it is very important to correct these by moving the head slightly on the mouthpiece, which is used as a guide to head position rather than as a rigid clamp. This done, the eye looks away for a few seconds and then returns rapidly to the desired fixation point. Adjustments of the colorimeter controls for a match are made and the eye looks away again. This glancing process is repeated until the measurement is satisfactory. Skill is soon acquired at head positioning and rapid and accurate fixation so that the period of time during which the eye is actually looking at the field can be increased somewhat with practice. If this period is allowed to become too long disturbing after-images develop which make several minutes rest an essential. A few minutes dark adaptation was allowed before any series of observations were made.

RESULTS

Luminosity curves. The luminosity curves for the three retinal positions are shown in Figs. 2 and 3. These curves, which are generally described as the equal-energy luminosity curves, record the reciprocal of the amount of energy at each wave-length required to produce a visual sensation of brightness of constant magnitude. The maximum of the curve for the central position has been arbitrarily adjusted to 100 for both observers and the curves for the other areas are shown with their ordinates adjusted to allow for the sensitivities of these areas relative to the sensitivity in the central position. The curves shown are smooth free-hand curves drawn so as to be the best fit to the means of the observations.

In the case of W.D.W. the curves were recorded using a type A field. This field was viewed centrally or in the 20′ or 40′ positions according to which curve was to be measured. The lower patch was illuminated by light of wave-length 0·530 μ. and maintained at a constant energy content throughout, while the upper patch was illuminated by various wave-lengths (λ) taken in turn through the spectrum. A brightness match between the two halves of the field was made by controlling the intensity of λ, and although there was in general a colour difference between the two patches, reasonably consistent observations were obtained. The luminosity curve was then derived by plotting the inverse of the energy required at wave-length λ for the brightness match. The relation between the curves for the three retinal areas was obtained by making a subsidiary observation with a type B field. The lower patch and one of the upper patches were illuminated by light of wave-length 0·530 μ. and brightness matches made between *a* and *a′*, *a* and *b′*, *a* and *c′* in turn, *a* being fixated in each case. From this observation the relative sensitivities of the three retinal areas to wave-length 0·530 μ. could be calculated and the relative heights of the three luminosity curves deduced. A similar type of observation was also made with 0·600 μ. and the curves again adjusted to be at the correct heights at this wave-length. These two sets of curves were very similar and from them a mean set of curves was calculated as shown in Fig. 2. The central curve is that published by Willmer & Wright (1945) for W.D.W.

In the case of L. C. T., Fig. 3, all the curves were measured with a type B field. The central curve was measured by a step-by-step method as follows: The wave-length of the lower beam was set at 0·530 μ. and adjusted to a convenient brightness. The upper beam was then set at 0·530 μ. and a brightness match made by moving the neutral wedge controlling the intensity of the upper beam. The wedge position was then recorded and with the upper beam set at wave-length 0·540 μ. three heterochromatic brightness matches were made and recorded. The wedge was then set at the mean value of these three recordings and the observer altered the lower beam in colour and brightness until equality was obtained. After this, the upper beam was set at 0·550 μ. and a further three heterochromatic matches made. Each wave-length was measured by a continuation of this process and from the mean wedge readings a figure proportional to the energy required to produce a visual sensation of constant magnitude was determined from the energy calibration curve of the instrument; the reciprocal of this energy then gives the relative luminosity.

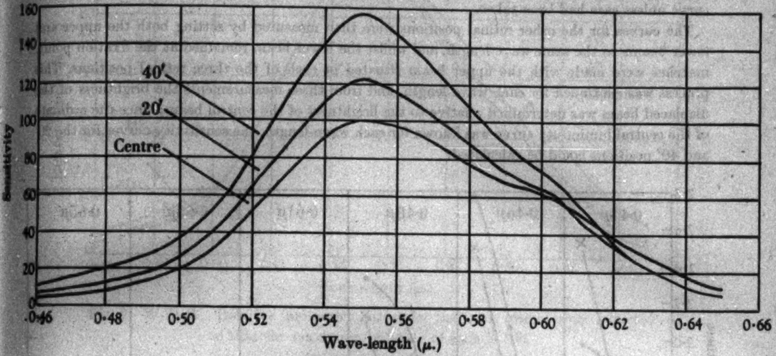

Fig. 2. Sensitivity curves for W. D. W.

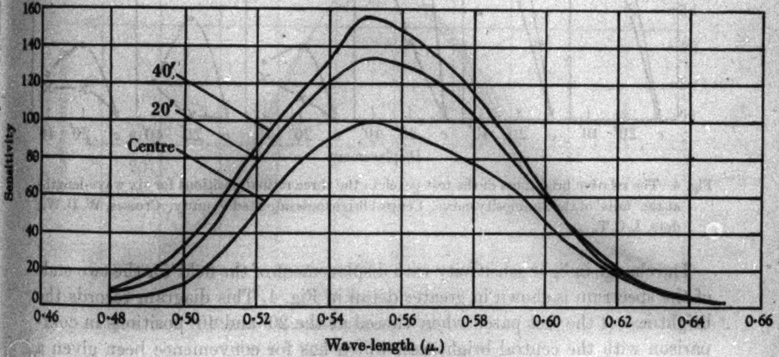

Fig. 3. Sensitivity curves for L. C. T.

In this way each wave-length was compared in brightness with an adjacent wave-length through the medium of a comparison field which did not differ greatly from it in colour. The advantage of this method over the more usual one, in which a comparison field of fixed wave-length is used, is that big differences of colour are avoided and for L. C. T., who found big colour differences very disturbing, the measurements were much improved in consistency in spite of the fact that errors of matching were cumulative and might have produced quite large discrepancies in the 'tails' of the curve unless care had been taken.

The curves for the other retinal positions were then measured by setting both the upper and lower beams to the same wave-length, and whilst the lower beam remained at the fixation point, matches were made with the upper beam situated in each of the three retinal positions. This process was continued for each wave-length, and from these measurements the brightness of the displaced beam was determined relative to the brightness of the central beam; since the ordinate of the central luminosity curve was known for each wave-length, the sensitivity curves for the 20′ and 40′ positions could be calculated.

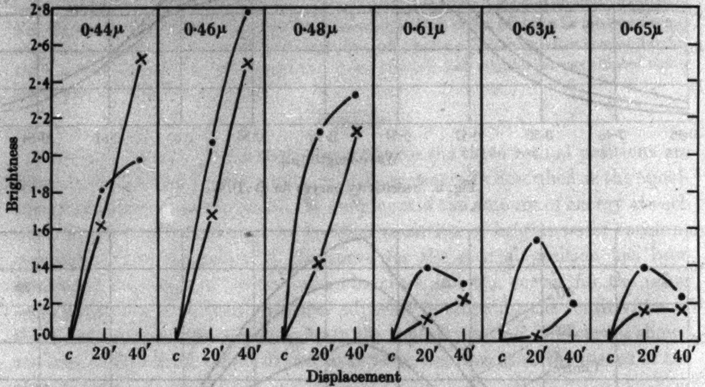

Fig. 4. The relative brightness of the test patch in the three retinal positions for six wave-lengths at the 'tails' of the luminosity curve. Central brightness adjusted to unity. Crosses, W. D. W.; dots, L. C. T.

The change in light sensitivity with displacement of the field for the two ends of the spectrum is shown in greater detail in Fig. 4. This diagram records the brightness of the test patch when viewed at the 20′ and 40′ positions in comparison with the central brightness, which has for convenience been given a value of unity for each wave-length. Results are shown for both observers, each of whom found a very marked increase in sensitivity to blue light as the patch moved away from the centre.

With regard to the accuracy of the observations, the mean of five readings, in Fig. 3, for the central curve for wave-length 0·550 μ. had a standard error of ±0·063. The standard error of the mean of the 20′ curve was ±0·035 (6 readings) and of the 40′ curve ±0·063 (6 readings). In Fig. 4 at 0·460 μ. the standard error at 20′ (L. C. T.) was ±0·168 (4 readings) and at 40′ ±0·229 (4 readings). The differences among the curves at the apices and on the blue side of the maxima in Fig. 3 are

clearly significant, but between 0·570 and 0·620 μ. there is probably no difference between the 20′ and 40′ curve, although they both differ from the central curve. Errors for W.D.W. are somewhat less than those stated above.

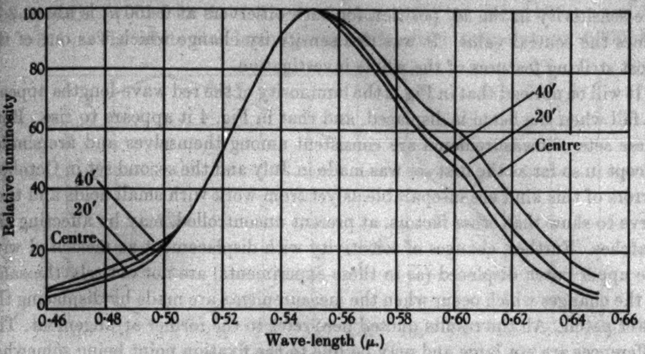

Fig. 5. W.D.W. Relative luminosity curves for the three retinal areas adjusted so that the maximum ordinate of each is 100.

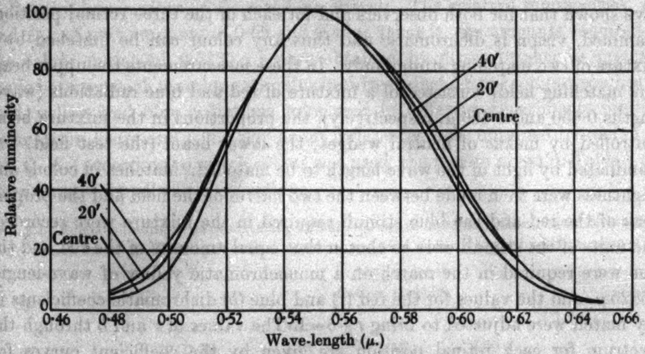

Fig. 6. L.C.T. Relative luminosity curves for the three retinal areas adjusted so that the maximum ordinate of each is 100.

Even though these two sets of curves have been measured by different methods, the similarity of shape between them is remarkable, as may be seen by comparing Figs. 5 and 6 which show the curves of Figs. 2 and 3 plotted so that the maximum ordinate of each curve is 100. Moreover the general shape does not greatly alter with displacement of the beam to the 20′ and 40′ positions and the small hump at 0·600 μ. in W.D.W.'s central curve is reproduced in his two other curves. When, however, the 20′ and 40′ curves bear their

correct relation to the central curve as in Figs. 2 and 3 it can be seen that fairly large increases in sensitivity are obtained at most wave-lengths when the beam is displaced; the greatest changes being in the blue region of the spectrum where the sensitivity in the 40′ position for both observers at 0·460 μ. is about 2·75 times the central value. It was this sensitivity change which was one of the most striking features of the whole investigation.

It will be noticed that in Fig. 2 the luminosity of the red wave-lengths appears to fall when the beam is displaced, and that in Fig. 4 it appears to rise. Both these sets of measurements are consistent among themselves and are similar except in so far as the first set was made in July and the second set in October. Errors of this kind are inseparable as yet from work with small fields and they serve to show that other factors, at present uncontrolled, may be affecting the matches. Further, changes of sensitivity with displacement as measured with the upper patch displaced (as in these experiments) are not entirely the same as the changes which occur when the measurements are made by displacing the lower patch. All the results quoted here refer to the former arrangement. The differences are not large and may be due to the fixation point being somewhat displaced from the anatomical centre of the fovea.

Dichromatic coefficients. With a type A field, colour-matching experiments have shown that for both observers and for each of the three retinal positions examined, vision is dichromatic and thus any colour can be matched by a mixture of two matching stimuli only. In these measurements the upper beam (the matching field) consisted of a mixture of red and blue radiations (wave-lengths 0·650 and 0·460 μ. respectively), the proportions in the mixture being controlled by means of neutral wedges; the lower beam (the test field) was illuminated by light of the wave-length to be matched. Matches in colour and brightness were then made between the two halves of the field and the proportions of the red and the blue stimuli required in the mixture were recorded. The units of the stimuli were so chosen that equal amounts of the red and the blue were required in the match on a monochromatic yellow of wave-length 0·5825 μ. and the values for the red (r) and blue (b) dichromatic coefficients in any match were adjusted to bring $r+b=1$. The values of r and b through the spectrum for each retinal position are given by the coefficient curves for W.D.W. in Fig. 7. It should be noted that the units for each retinal position were based on a 0·5825 μ. match made in that position; hence all the curves have coefficients of 0·50 at the wave-length 0·5825 μ. Similar curves for L.C.T. were also obtained.

The advantage of evaluating the coefficients in this way has been discussed elsewhere (Wright, 1929; Pitt, 1935); the main advantage, however, is that the coefficients as measured on this basis are independent of macular pigmentation. The differences in the curves of Fig. 7 can therefore be attributed to differences in the sensitivity of the receptor processes themselves.

The white point (i.e. the wave-length which could be matched with white) for each of the three retinal positions was measured. The result for the central position for W.D.W. was 0·578 μ.; for 20′, 0·575 μ.; and for 40′, 0·572 μ. The corresponding values for L. C. T. were, centre 0·577 μ.; 20′, 0·576 μ.; and for 40′, 0·571 μ. The white standard S_B was used, colour temperature, 4,800° K.

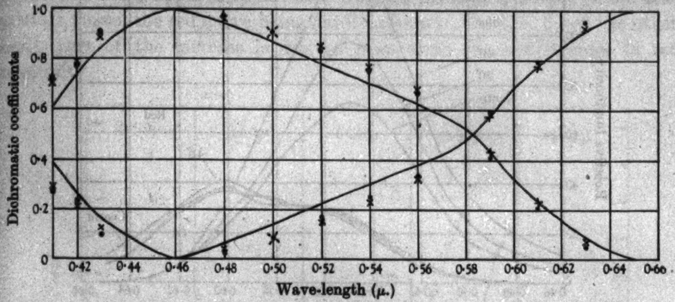

Fig. 7. W.D.W. Dichromatic coefficients. Full curve, central; crosses, 20′; dots, 40′.

Relative luminosities of the matching stimuli. The relative luminosities, L_R and L_B, of unit amounts of 0·65 and 0·46 μ. (that is, the amounts required in the colour match of 0·5825 μ.) were obtained by direct brightness matches made between each matching stimulus in turn against a fixed comparison beam. Red, yellow and blue radiations were used in different experiments for this comparison beam and while significant differences were obtained by one of us (W.D.W.) according to which colour was used, the results given in Table 1 are the averages for all the observations.

Table 1. Relative luminosities of matching stimuli. (Units of matching stimuli based on 0·5825 μ. match for W.D.W. in each retinal position, and for L.C.T. in central position only)

	L_B/L_R Retinal position		
Observer	Centre	20′	40′
W.D.W.	1·37	1·47	2·00
L.C.T.	1·30	1·93	2·93

Spectral mixture curves. The unit dichromatic equation for wave-length λ is given by

$$C_\lambda = r_\lambda R + b_\lambda B,$$

where r_λ and b_λ are the dichromatic coefficients for that wave-length. The equality match between C and the R, B mixture can also be expressed in luminosity units by applying the relative luminosity values of R and B as given in the previous section. Thus

$$L_\lambda C_\lambda = r_\lambda L_R R + b_\lambda L_B B,$$

where L_λ is given by $L_\lambda = r_\lambda L_R + b_\lambda L_B$.

The ordinate F_λ of the equal-energy luminosity curve at wave-length λ can be divided into two parts representing the amounts in luminosity of 0·65 and 0·46 μ. which are equivalent to the amount of λ present in the equal-energy spectrum. These amounts can be calculated as follows:

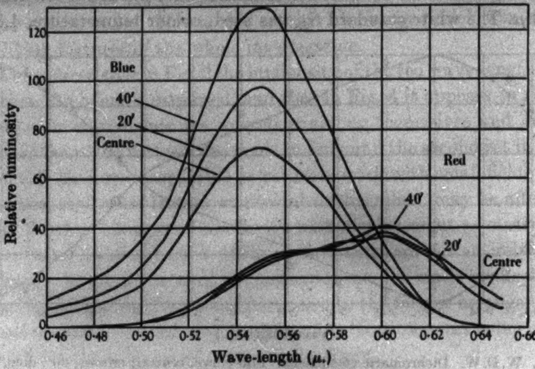

Fig. 8. W. D. W. Spectral mixture curves.

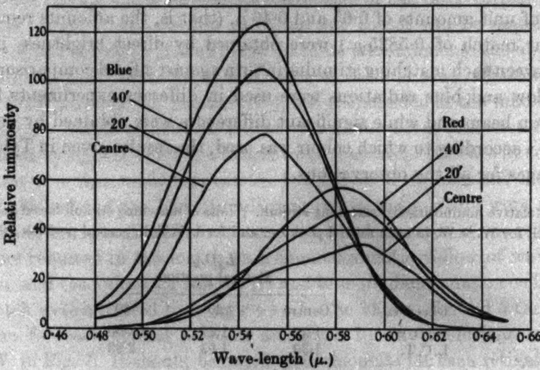

Fig. 9. L. C. T. Spectral mixture curves.

$$\text{Amount of } R = \frac{F_\lambda}{L_\lambda} r_\lambda L_R,$$

$$\text{Amount of } B = \frac{F_\lambda}{L_\lambda} b_\lambda L_B.$$

If these values are worked out for various wave-lengths through the spectrum, the results can be plotted to give the mixture curves shown in Figs. 8 and 9. These curves were derived from the luminosity curves of Figs. 2 and 3, and therefore include the effect of the change in light sensitivity with retinal position.

In the case of W.D.W. the increase of total luminosity with displacement of the test beam apparently develops from an increase in height of the blue mixture curve, the red curve being hardly affected. For L.C.T., on the other hand, part of the increase is derived from some absolute increase in red sensitivity.

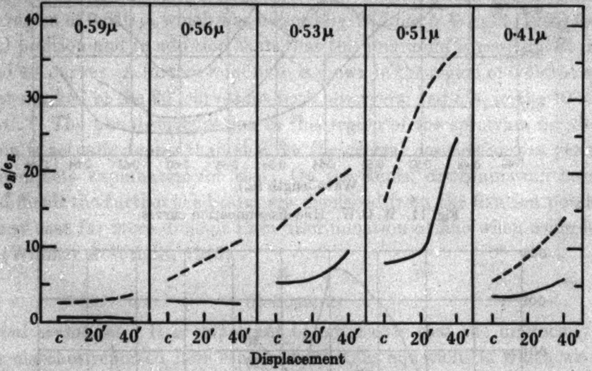

Fig. 10. Ordinate: the energy ratio e_B/e_R of the blue stimulus to the red stimulus required to match the test colour, when the mixed field is viewed always in the central position. Abscissa: displacement of the test field. Dots, W.D.W.; full curve, L.C.T. For explanation, see text.

The change in colour sensitivity with foveal position can also be expressed in terms of the energy ratio e_B/e_R of the blue stimulus to the red stimulus required to match the test colour, when the mixed field is viewed always in the central position, while the test beam is viewed in turn in the central, 20′ and 40′ positions. The change in this ratio with retinal position of the test field is illustrated in Fig. 10 for various wave-lengths of the test field; these curves have, however, been derived by calculation from the data in Figs. 2, 3, 7, 8, 9 and Table 1, and not by direct observation. This method of expressing the results has the advantage that the change in colour of the test field is shown in terms of matching stimuli viewed on the same area of the retina in all cases, so that the matching stimuli are of constant light and colour quality. The very striking increase in the e_B/e_R ratio, particularly at 0·51 μ., was the quantitative counterpart of a subjective change in the test field with change in retinal position; thus, 0·51 μ. viewed centrally looked green but in the 40′ position turned blue.

Hue discrimination. The hue discrimination of both observers was measured with a type A field. The upper beam was set at wave-length λ and the lower beam adjusted to wave-length λ ± Δλ until a just noticeable difference of colour could be seen. Owing to the very large wave-length difference recorded in many of the observations, it was frequently convenient to measure the step on one side of λ only, the step generally being observed towards the yellow part of the spectrum. The intensity of the lower beam was fixed at a suitable level and equality of intensity between the two beams was maintained throughout the observations by adjusting the neutral wedge in the upper beam. The ability to discriminate between neighbouring wave-lengths is inversely proportional to Δλ, and in Figs. 11 and 12 Δλ is plotted against λ ± ½Δλ. Δλ is always considered as positive. The central curve for W. D. W. is slightly modified from the curve published by Willmer & Wright (1945).

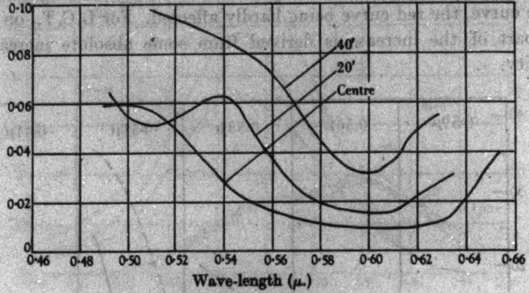

Fig. 11. W.D.W. Hue-discrimination curves.

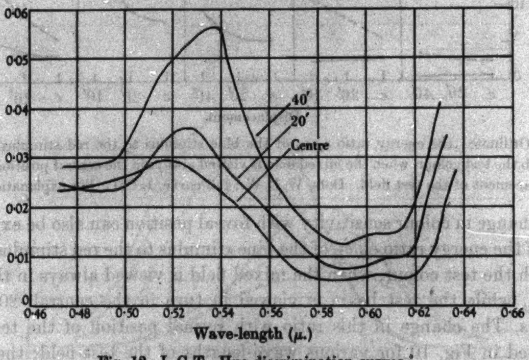

Fig. 12. L.C.T. Hue-discrimination curves.

As regards the accuracy of the observation, for wave-length 0·490 μ. and for L.C.T., the standard error of the mean of observations taken at the centre was ±0·0021 λ. (6 readings). For the 20′ position the error was ±0·0036 λ. (6 readings), and for the 40′ position ±0·0034 λ. (6 readings). From these figures it might be argued that the difference between the central and 20′ curve shown at this point is not significant, but these errors are mainly produced by day-to-day variations in the results as a whole and these curves have been judged significantly different by an examination of their relationship within the results of each day. The day-to-day variations are probably due to a variable interpretation of the words 'just noticeable difference' and this is also the probable explanation of the fact that any step made by L.C.T. is approximately half that made by W.D.W. under similar conditions. Nearer the minimum of the curve the standard error is only ±0·0005 λ. for the 20′ curve.

These curves again show the minimum of hue step (maximum discrimination) in the region of 0·610 μ. which was noticed by Willmer & Wright (1945) for the central position and in addition show that this minimum is reproduced in the 20′ and 40′ curves. A further minimum is shown in the region of 0·490 μ. which is most marked in the 20′ curves for both observers, and also in the 40′ curve for L.C.T. The hue discrimination in this region of the spectrum for the 20′ position is actually better than that for the central position and as yet there is no complete explanation for this. On the whole, discrimination becomes more difficult the further the beams are displaced from the fixation point and is in any case far more difficult than discrimination of hue when using larger fields (Willmer & Wright, 1945).

DISCUSSION

Visual sensations. It is important to emphasize that the luminosity and colour matches reported here represent stimulus equations in which we have had no concern with the quality of the sensations produced by the stimuli apart from establishing equality between the two halves of the field. We shall not, for example, discuss whether the 0·46 μ. matching stimulus gives rise to a blue or a green sensation, or possibly to one sensation under one condition and another under a different condition, and although our results may be related to the spectral sensitivity of the receptor mechanisms in the retina, no study of the sensations aroused by the activity of these receptor mechanisms has been made. If it is true that there are two receptor processes active within the central fovea (Willmer & Wright, 1945), the further problem arises of how the various colour sensations can be stimulated with only two such processes responding in any small retinal area. Before this question can be settled, a different type of experiment from that described in this paper is required, in which the quality of the sensation is studied for various intensities and wavelengths of the light stimulus, for various sizes of test spot and retinal position and, most difficult of all, for various conditions of fixation, e.g. momentary fixation, prolonged fixation, no fixation, etc.

Anatomy. Since special steps have been taken in this investigation to neutralize the chromatic aberration of the eye, a great increase in the precision of retinal stimulation has been achieved and some account of the anatomy of the central fovea is necessary in order to interpret the results. All the facts and the outlines of Fig. 13 have been taken from Polyak (1941). The figure, which represents a cross-section of the central fovea, shows the relationship between the image of the circular field and the receptors. Linear measurements can be judged from the scale of μ. below the diagram and the scale of minutes below this shows the visual angle to which the linear measurement corresponds. The positions and extent of the image of the circular field in the photoreceptor layer correspond to the positions shown at the foot of the figure.

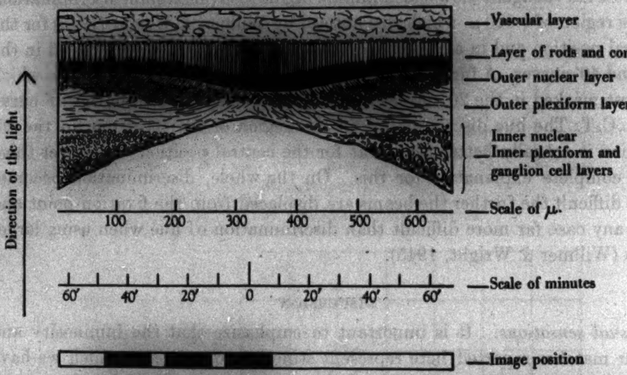
Vascular layer
Layer of rods and cones
Outer nuclear layer
Outer plexiform layer
Inner nuclear
Inner plexiform and ganglion cell layers
Direction of the light
100
200
300
400
500
600
Scale of μ.
60′
40′
20′
0
20′
40′
60′
Scale of minutes
Image position

Fig. 13. The relationship between the image of the test patch and the photoreceptors of the retina. (The diameter of the more lateral cones has been somewhat exaggerated.) (Modified from Polyak.)

The thinning and disappearance of the ganglion and inner nuclear and plexiform layers, which occur in this region, can be seen and also the increase in length of the cones at the foveal centre. This increase in length is associated with a decrease in the diameter of the inner segments of the cones and in the foveal centre this diameter is 1·5 μ. as compared with 2·5 μ. in the more lateral positions. The yellow macular pigment is confined to the ganglion and inner nuclear layers and is, therefore, most evident in the foveal slopes where these layers are increased in depth. The foveal centre contains little or no pigment.

Polyak states that the area containing the slender cones at the centre of the fovea usually occupies a visual angle of 10′ on either side of the central point and if one assumes that the fixation point is at the centre, the circular field in its central position would lie wholly on the slender cones, and it has been estimated that the image of each half of the circular field would then stimulate as few as 700 of these cones. The 20′ and 40′ areas fall on the somewhat fatter cones of the more lateral part of the fovea and here the image of each half of the field stimulates somewhat less than 700 cones. It is unlikely that any rods are included in the 40′ area, for Polyak states that even in a small fovea, 50′ of visual angle on each side of the centre is absolutely free from rods and that even at 1° from the centre there are only a few rods. From this it would follow that all three field positions that we have used are free from the influence of the rods. It is interesting to note that the ability to resolve the field became rapidly worse as the field was displaced and that this might be due either to the introduction into the beam of the bodies of a few ganglion cells, to the increased diameter of the foveal cones which occurs in the 20′ and 40′ positions, or more probably to the less precise representation of each cone in the cerebral pattern.

Dichromatic vision. In so far as vision for all our image positions was dichromatic, these experiments confirm, for the central area, the findings of Hartridge mentioned earlier in this paper that the size of the image and not its retinal position determines whether vision shall be dichromatic or trichromatic. But although vision was dichromatic for each position (which may mean that the receptor mechanisms are similar in kind all over the fovea) very striking increases of sensitivity occur in the blue region of the spectrum with displacement of the test beam, so that, whatever the receptor mechanisms may be, they clearly function in a way which is peculiar to the retinal position. It is possible to obtain subjective confirmation of this non-uniformity of behaviour from matches made with the more extended field of a trichromatic colorimeter. There is in fact a central pip in the fovea which is markedly less sensitive to blue light than the immediately surrounding zones. This pip can be observed as a small spot of different colour when a blue-green field is being matched.

This variation of blue sensitivity may also explain the observation made by König (1894) and by Willmer & Wright (1945) that a dichromatic match was at once disturbed unless perfect fixation was maintained. Without the aid of fixation spots, the image of the test patch would only remain stationary upon the retina and so give sufficient time for a match to be made, when it lay at the foveal centre. Since no other retinal area can be steadily directed towards the test patch of light, its image would, elsewhere on the retina, be at all times on the move across a retina of varying sensitivity, so that no test for dichromasy could be made, although vision might nevertheless be dichromatic for small fields all over the retina. Our experiments indicate that this explanation might be correct; when fixation spots were provided, it was confirmed that in addition to the centre, the vision of the lateral parts of the central fovea was also dichromatic. It may be that further experiment will show that the vision of the whole of the retina is dichromatic when small fields are used.

Trichromatic vision. If any small field within the fovea gives dichromatic vision, why should a larger field be trichromatic? One answer may be that, because of the differences in the dichromatic mixture curves for different foveal positions, satisfactory colour matches at two or more points at the same time can only be secured if three matching stimuli are used. This would mean that the apparent trichromasy of the retina was an artefact due to the varying spectral sensitivity of one or both of the receptor processes across the retina. On the other hand, it may be that there are only two types of receptor at the centre and a third in the more peripheral parts of the fovea, but that owing to the poor discrimination in these regions when small fields are used, apparently satisfactory colour matches can be made with only two matching stimuli.

Foveal insensitivity to blue light. Polyak (1941) states that each bipolar cell supplying the foveal periphery has a larger number of receptors in synaptic contact with it than a corresponding bipolar cell supplying the foveal centre. The same is true for the ganglion cells, whose axons are the fibres of the optic nerve, and it might be argued that this increased complexity of connexion in the foveal periphery might enable the response of one receptor to sum with that from adjacent receptors and so produce the greater sensitivity to light noted in these experiments. An explanation along these lines, however, cannot be wholly correct because the retinal neurons are united into an anatomical whole by the presence of the horizontal neurons and, as in other parts of the central nervous system, the results of stimulation of the receptors will depend upon the properties of the synapses between the neurons. These properties are as yet scarcely investigated and it may be that when they are known, the true explanation of the increase of sensitivity will be apparent.

Another explanation of the blue insensitivity of the foveal centre is that it is not due to an alteration of the receptor sensitivity but that the yellow macular pigment preferentially absorbs the blue and violet rays. Since the pigment is concentrated in the foveal slopes, this cannot be correct: the centre in our experiments shows greater insensitivity to blue than the 40′ position and in neither is very much pigment present. If any is present, the 40′ position should be less sensitive to blue than the central position.

SUMMARY

1. Luminosity, dichromatic coefficient and hue-discrimination curves have been measured with a 15′ test field and spectral mixture curves calculated for two observers and for three retinal positions of the right eye, central, 20′ and 40′ displaced into the nasal half of the visual field. The colorimeter developed by one of us (Wright, 1927, 1939) has been used.

2. The luminosity curves are similar in shape but show that there are fairly large increases in sensitivity to blue light as well as moderate increases elsewhere in the spectrum.

3. The spectral mixture curves show that the increase of sensitivity with displacement in W.D.W. largely develops from an increase in height of the blue spectral mixture curve, whereas for L.C.T. there is also some increase in the height of the red curve.

4. All three hue-discrimination curves show a minimum hue step in the region of wave-length $0{\cdot}610\,\mu$. and the curve for the 20′ position in both observers shows a second minimum at $0{\cdot}490\,\mu$., which is below the central value for this wave-length.

5. Since steps had been taken to render the eye achromatic, the precise relationship of the image of the test patch to the retinal receptors has been discussed.

6. The nature of the vision at the central fovea is considered and it has been suggested that the normal trichromasy of foveal vision, when large fields are used, could be an artefact and that the third matching stimulus allows satisfactory matches to be made at two or more points at the same time.

We wish to express our thanks to Miss M. Gilbert for recording the observations and to the Medical Research Council for their continued support for visual research at Imperial College.

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THE EFFECT OF LIMB POSITION IN SEATED SUBJECTS ON THEIR ABILITY TO UTILIZE THE MAXIMUM CONTRACTILE FORCE OF THE LIMB MUSCLES

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It is well known that human muscles in maximum contraction exert a large force in the line of the tendon, often exceeding 1000 lb., and that this force is dependent on the initial length of the muscle as shown by Evans & Hill (1914), Reijs (1921), and others. In everyday life these forces are not noticed, for the muscles work at a mechanical disadvantage, usually of about 10 : 1, which gives a high gear-ratio and allows rapid movement. The precise mechanical disadvantage is dependent on the angle of the acting joint and this finally determines the force which can be exerted.

In standing subjects, Vernon (1924), and Bedford & Warner (1937), observed the effects of posture on the pull which can be exerted on a dynamometer handle; Garry (1930) showed the importance of limb position in determining the pull and push on a hand-lever moving in a vertical plane. In seated subjects, similarly, the force exerted (measured at right angles to the tibia) by knee-joint extension was recorded by Duboux (1942) and Haxton (1945*a*) when investigating the function of the patella; Franke (1920) recorded the effect of elbow-joint extension on the force exerted by the triceps, biceps and brachialis muscles.

A particular case of the relationship between force exerted and angle at the joint, which is important in industry, is given when a seated subject pushes on a foot-pedal or hand-lever and makes use of a seat back-rest to push against; the force is exerted along the line of the tibia or radius, and the placing of the pedal or hand-grip relative to the seat, which determines the joint-angles and the force obtainable is highly critical. This forms the subject of the present investigations which were started originally for the purely practical purpose of placing controls; on this account the results of pushing on a hand-grip are compared with pulling for the same positions. The results seem of interest in relation to the mechanical problem of utilizing the force developed by muscle.

PLAN OF EXPERIMENTS

In order to ascertain, in general, the influence of position on the force that can be exerted on an isometric control by a seated operator, and yet to limit the scope of the work, the experiments were arranged as follows:

Maximum exertable push was measured on:

A foot-pedal placed in front of a seat and in the same vertical plane as the hip-joint of the experimental subject. The pedal was mounted in different positions so that for each of five different angles of thigh to the horizontal (referred to as angle α) the relation between push exertable on the pedal and knee-angle (referred to as angle β) was found (Fig. 1*a*).

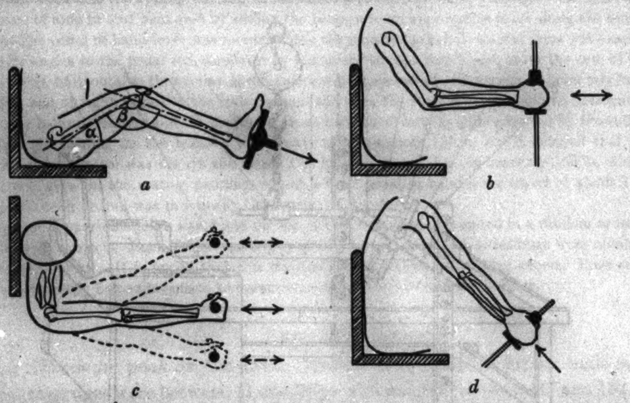

Fig. 1. Diagram illustrating the different conditions under which the maximum force that could be exerted by a seated subject on foot and hand controls was determined. For details, see text.

A hand-lever mounted in front of a seat so that horizontal push was recorded at different distances of hand-grip from the seat back. The grip on the lever was kept at a constant height of 15 in. above the seat, and the experiment was done with the lever moving in each of three different vertical planes, passing through the mid-line of the body, through the shoulder-joint, and 15 in. lateral to the shoulder-joint (Fig. 1*b*, *c*).

Maximum pull exertable on the hand-lever was found for:

A horizontal pull with the subject keeping his back against the back-rest. In this case not only was the effect of distance of the grip on exertable pull measured, to compare with pushing for the three vertical planes described above, but also a study was made on the effect on the pull of having the actual grip on the vertical hand-lever at different heights, and also of changing its axis from a vertical to a horizontal plane (as in rowing).

An upward direction of pull at 45°, in this case with different degrees of hip-flexion so that the back was bent forward from the seat back-rest (as in pulling on a car hand-brake), but only with the lever moving in a to-and-fro vertical plane passing through the shoulder-joint (Fig. 1*d*).

APPARATUS

The apparatus used for obtaining most of the above results is shown in Fig. 2. A metal seat (*s*), with a flat, padded, and adjustable back-rest, which supported both the subject's pelvis and back, was mounted in a rigid wooden frame (*f*) fixed to the wall and floor; two upright beams (*b*) could be bolted securely in any of eight positions along the frame. An axle (*a*) of 2 in. diameter steel tubing could be held so as to rotate freely in any of the ten bearing-holes shown in the upright beams. A 300 lb. spring balance (*m*), suspended from a ceiling beam, was attached by a chain (*c*) to a hooded collar (*o*) fixed on a steel rod (*r*). The latter was inserted through a hole in the axle at right angles to its length, and held there by two collars (*d*).

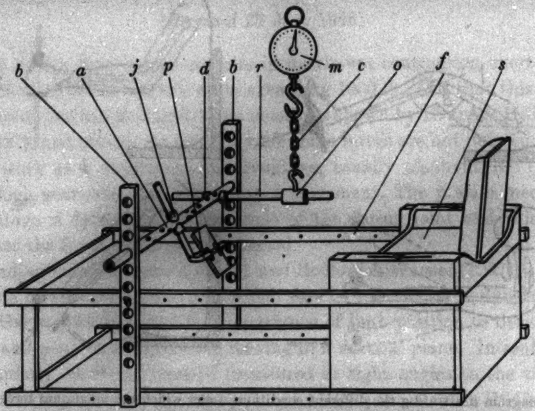

Fig. 2. Diagram of frame apparatus which was modified for use in the different experiments described. For details, see text.

Into any appropriate one of a series of holes in the axle (*a*) was inserted either a pedal (*p*) or a hand-lever (not shown in the figure) and this was prevented from rotating by grooved collars (*j*). The pedal had a board freely slung under its cross-bar. The hand-lever was a steel rod on which a wooden hand-grip could be clamped by collars at any height above the axle; the lower collar was large enough to form a rest for the hypothenar eminence of the hand; this ensured that the grip was always held in the same place and allowed the turning moment about the axle to be measured.

A simpler form of apparatus than the above was used for the experiments on horizontal pulling only. It consisted of the 300 lb. balance with a hand-grip attached by a short length of chain. The balance was held by a hook which could be pinned at any desired height on a vertical beam fixed to a wall. On the floor in front of the beam four parallel white lines were marked at right angles to the wall and each white line was divided into divisions of 6 in. The divisions allowed a seat, of the same height as that on the first apparatus, to be placed at any distance from the grip when the latter was pulled, while the white line used determined the vertical plane of the grip relative to the seat.

METHODS

(*a*) *Measurement of joint-angles.* Angles were measured with a long-armed hinged protractor, which carried a plumb-line when necessary. Angles were taken from bony points; for example, in the leg, a projecting point on one protractor-arm was laid against the greater trochanter of the femur while the outer end of the protractor-arm was against the lateral epicondyle. Similarly, for the lower leg, the fibula head and lateral malleolus were the bony points used. Angles were measured both with the subject at rest and when maximum force was being exerted.

(*b*) *Measurement of maximum force.* In the frame apparatus, the axle was mounted in the desired position and the rod for balance attachment kept horizontal for any axle height by fixing its collar-hook into the appropriate link of the balance-chain. The chain was kept vertical for any distance of axle to seat back-rest by sliding the balance to an appropriate place along the ceiling beam. The pedal or hand-lever was mounted into the required axle-hole so that force was exerted at right angles to the pedal rod, as shown by the freely pivoted foot-board, or, in the case of the hand-lever, horizontal to the vertical lever placed in the required to-and-fro plane. Since any force applied was at right angles to the rods through the axle, the force exerted could be determined from the balance reading and the lengths of these rods; these lengths were adjusted by the collars so that the reading on the balance was always approximately 150 lb. which ensured that the travel of the control was always the same. The balance moved $\frac{3}{4}$ in. in reaching 150 lb. which, with the 'give' in the seating padding, meant a total pedal or hand-lever travel of about 1 in. Thus the push or pull was practically 'isometric' in character.

Maximum exertable force was found for the different positions presented in a random order to the various subjects. Each subject took long rests between efforts, while readings were obtained on other subjects. It is hoped that this minimized any fatigue or training effects. Prize competitions greatly helped incentive to ensure maximum effort from every subject.

RESULTS

(*a*) *Maximum push on foot-pedal.* Results were recorded for six male subjects; their ages were between 21 and 36 yr. and weights between 147 and 182 lb. For a given foot-pedal position the variations between readings for maximum push of an individual were very small compared with the physical differences between individuals: each subject recorded at least three maximum efforts, and the mean of two similar readings was taken. It should be noted that throughout this article *twice* the S.E. of means is given, representing less than 1 in 20 probability of the mean differing from its stated value by more than this amount. In order to compare one subject with another the seat was adjusted, to allow for differences in leg length, so as to give the same joint-angles at maximum push.

The results are given in Table 1. These are for the right leg; only in some individuals was there a difference in the power of the two legs, whilst the mean pushes of the six subjects showed no significant difference between legs.

From Table 1 the graphs in Fig. 3 have been drawn; these graphs illustrate how, for any given thigh-angle α, the exertable push increases with increasing knee-angle β, which occurs as the pedal is moved away from the seat on an arc with the knee-joint as centre. Curves 1, 2 and 3 show how this rise in possible push becomes increasingly rapid until a well-defined 'limiting angle' is reached at 160° for angle β; above this angle push suddenly decreases, the pedal subjectively feels too far away and pushing becomes uncomfortable, if not dangerous, for the knee has a tendency to go over 'top-dead-centre' and flick straight. Curves 4 and 5 necessarily stop as shown, well before the limiting angle is reached, for with the great degree of hip-flexion which they represent the knee cannot be extended further.

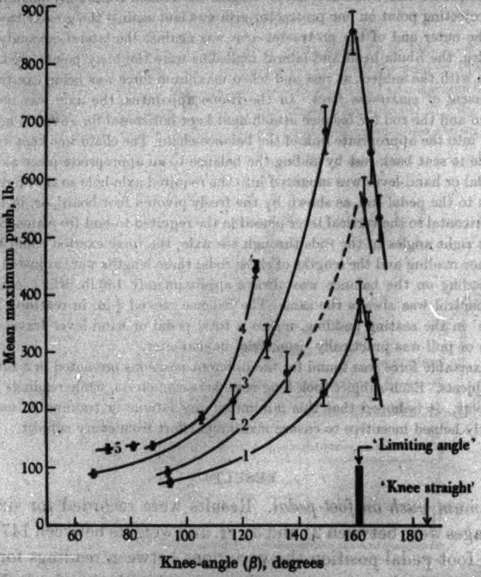

Fig. 3. The mean maximum push ($\pm 2 \times$ S.E.) exertable by six subjects on an isometric pedal placed in different positions that allowed different degrees of knee-extension (angle β) for each of five different angles of thigh to the horizontal. (Curve 1, thigh-angle (α) between -15 and -6°; curve 2, between $+5$ and 10°; curve 3, 15–19°; curve 4, 33–36°; curve 5, 48–49°.) Results as in Table 1.

The position, resulting in a thigh-angle α of 15° and knee-angle β of 160°, for which the greatest push of $845 \pm 35{\cdot}4$ lb. (*twice* S.E. of mean for six subjects), was recorded, is attained for the 90% range of adult male population when an isometric pedal-bar is placed 37·5–44 in. from a seat back-rest and 6–8 in. above the seat level. Seat adjustment to obtain these particular leg-angles

Table 1. Mean maximum push exertable by six subjects on an isometric foot-pedal in positions allowing different degrees of extension of the knee-joint (angle β) for five approximately constant values of thigh-angles to the horizontal (angle α)

Mean leg-angles recorded during maximum push (degrees)		Mean maximum push of six subjects ± 2 × S.E. (lb.)
Thigh-angle (α)	Knee-angle (β)	
− 6	94	73 ± 4·0
−15	149	227 ± 20·8
−10	162	385 ± 33·0
−10	165	346 ± 20·2
− 9	167	250 ± 34·4
+ 8	93	87 ± 6·7
10	136	270 ± 30·6
5	164	559 ± 35·4
19	67	89 ± 5·4
17	117	212 ± 28·0
16	129	319 ± 27·8
17	151	684 ± 38·6
15	160	845 ± 35·4
15	166	640 ± 57·2
15	169	530 ± 38·6
36	88	135 ± 5·2
33	106	184 ± 8·6
34	125	443 ± 44·6
48	72	133 ± 7·2
49	81	130 ± 7·0

Table 2. The mean maximum horizontal push on an isometric vertical hand-lever at different distances from the seat back-rest; for the lever in four different planes but with the hand-grip at a constant height of 15 in. above the seat level

	Distance from centre of hand-grip to seat back	Mean maximum horizontal push ± 2 × S.E. (10) (lb.)	
Vertical plane of lever	(in.)	Subject 1	Subject 2
1. Left shoulder (using left arm)	17	64·3 ± 3·8	57·5 ± 2·0
	23	83·7 ± 2·9	61·3 ± 3·4
	29	111·1 ± 4·2	77·6 ± 3·8
	33	93·7 ± 2·9	67·2 ± 3·9
2. Mid-line (using right arm)	17	98·9 ± 4·0	46·1 ± 2·5
	23	107·4 ± 7·1	57·6 ± 5·0
	29	116·3 ± 6·0	93·9 ± 5·1
	33	99·5 ± 1·9	77·0 ± 2·3
3. Right shoulder (using right arm)	17	99·2 ± 4·1	54·5 ± 1·8
	23	109·0 ± 4·6	60·9 ± 2·5
	29	126·6 ± 4·1	91·9 ± 4·6
	33	107·8 ± 7·0	72·6 ± 2·6
4. Outside shoulder 14 in. to right of mid-line (using right arm)	17	64·8 ± 4·7	—
	23	85·8 ± 4·7	64·0 ± 3·8
	29	108·2 ± 4·0	74·4 ± 4·0
	33	106·3 ± 3·7	66·5 ± 2·5

for maximum push is highly critical, to within 1 in., in order to obtain the greatest degree of knee-extension without exceeding the limiting angle. It must be emphasized that the six experimental subjects were powerfully built and are not a representative sample of the population. A random sample of thirty-two drivers of the Royal Armoured Corps exerted 691 ± 66·0 lb. (32) and of sixteen London schoolboys, aged 14–18 yr., exerted 688·75 ± 62·3 lb. (16) on a pedal in this position.

The results, in general, agree well with those of a similar investigation made by Müller (1935). There is disagreement in angles quoted for the position allowing greatest push. Müller quotes less precise angles and, although the thigh-angle of 15° may coincide with his estimate of between $-10°$ and $+24°$, the knee-angle β of 160° does not agree with his findings of 130–150°. This point is discussed later.

(*b*) *Maximum horizontal push on hand-lever.* For this, and all subsequent experiments, only two of the six subjects were used. Their weights were 168 and 172 lb., ages 27 and 26 yr., heights 71 and 70 in. respectively. Their arm lengths were identical, so that a hand control placed in the same position relative to the seat for each of them resulted in the same arm-angles and consequently comparable efforts. For convenience, distances of control to back-rest rather than angles were recorded. Each subject made two maximum efforts for each position in all planes, and this was repeated in random order for 5 days; thus, means recorded are of ten readings, and scatter is from variations in the individual and not from subject-to-subject, as in the pedal experiments.

The results are given in Table 2, and should be compared with those for pushing on the foot-pedal. As for the latter, these results show an increase in the exertable push as the lever is moved away from the back-rest, allowing greater elbow-joint extension, and this reaches a maximum at 29 in. after which, as for the knee, though to a lesser extent, the elbow has the same uncomfortable tendency to go over 'top-dead-centre' and straighten, so that the possible push decreases to the value for a straight arm at 33 in. Graphically, the form of the results is the same, only the absolute values for the arm are less. Push exertable with the right arm when the lever is moved in the mid-line plane is not significantly different from that exertable when it is moved in a plane passing through the right shoulder-joint, but it is less when this plane is outside the shoulder. In both subjects the left arm is weaker than the right.

It is interesting to note that both these subjects, and five others also who were asked to try, found pushing on a hand-lever under these conditions subjectively most tiring and unpleasant, mainly from an inevitable sense of raised intrathoracic tension, associated with difficulty in avoiding holding the breath; this effect is in no way apparent when pushing against the back-rest on a foot-pedal, or when pulling on a hand-lever in identical positions.

(*c*) *Maximum horizontal pull.* The procedure has been stated, except that obviously in this case the type of foot-rest determines the magnitude of the pull. In these experiments the subject's feet were flat on the floor and resin was applied to prevent slip, for if no slip occurred during a pull all findings should be comparable.

The results are given in Table 3 for the right arm with the plane of pull through the shoulder only. It will be seen from this table that, like pushing, horizontal pull is mostly dependent on, and increases with, extension of the elbow. However, possible effort does not, in this case, suddenly rise just before straightening of the elbow but increases steadily right up to full extension.

TABLE 3. Mean maximum horizontal pull on an isometric hand-lever for different heights and distances of the latter in relation to a seat. All results are for the right arm with the to-and-fro plane of pull through the shoulder

Height of centre of grip above seat level (in.)	Distance from centre of grip to seat back (in.)	Mean maximum horizontal pull ± 2 × S.E. (10) (lb.)	
		Subject 1	Subject 2
3	11	29·4 ± 1·0	22·0 ± 5·5
	17	37·8 ± 5·0	31·5 ± 3·4
	23	51·7 ± 5·0	45·7 ± 3·9
	29	59·5 ± 3·9	58·3 ± 6·2
	35	67·6 ± 3·5	60·6 ± 6·3
9	11	36·7 ± 4·4	30·9 ± 5·1
	17	48·0 ± 5·0	40·2 ± 6·2
	23	65·4 ± 3·0	56·7 ± 4·2
	29	71·2 ± 5·0	67·5 ± 7·4
	35	80·0 ± 5·0	74·0 ± 6·2
15	11	41·5 ± 4·5	33·6 ± 3·2
	17	57·4 ± 3·9	50·4 ± 4·3
	23	67·3 ± 4·1	57·9 ± 5·0
	29	74·8 ± 3·4	63·8 ± 9·6
	35	77·6 ± 3·0	72·2 ± 4·3
21	11	47·8 ± 4·0	37·5 ± 4·5
	17	61·1 ± 3·3	56·1 ± 3·3
	23	65·9 ± 3·1	63·6 ± 3·2
	29	66·1 ± 2·2	65·5 ± 3·7
	35	74·7 ± 2·2	72·3 ± 2·3

There is no limiting angle. It should be noted when comparing the results of pulling and pushing that, as one tends to pull a grip with the flexed fingers but push with the palm, distances for corresponding points are about 2 in. more in the former than in the latter; thus the arm was fully extended in the two cases with the grip at 35 and 33 in. from the back-rest respectively. For comparable positions horizontal pull is always less than push.

From the practical aspect, the results show also how exertable pull is dependent on the height of the grip itself. For horizontal effort a grip cannot be placed more than about 3 in. below, nor more than 24 in. above seat level owing to the limits of abduction and adduction at the wrist-joint.

It will be seen that at heights of grip of 9 and 15 in., between which there is no significant difference, the greatest pulls are possible. This corresponds roughly to a grip placed at the height of the elbow-joint when the upper arm is vertical; this was subjectively the most comfortable height.

In Table 3 the axis of the grip itself was in the vertical plane but tilted forward at 20° to the vertical to conform with the anatomical axis of the clenched fist; all the results were repeated with the grip axis horizontal and the hand pronated, as in rowing, and in 85% of the total 800 pulls the exertable force was less. This difference in the two subjects is significant but slight, making on an average less than 10% difference in exertable pull for any position.

As for pushing, changing the vertical plane of the lever from one through the shoulder to one through the mid-line made no significant difference to the exertable pull, but the latter was less when the plane of the lever was outside the shoulder. It is evident, in the latter case, that the subject may either pull obliquely or, by flexing his elbow and abducting his upper arm, pull with his forearm in two planes at right angles to the lever. The former procedure was investigated on the frame apparatus, the latter with a direct pull on the balance, as it was certain that so long as the chain was kept in the correct vertical plane the second type of pull only could be given. With the to-and-fro plane of the lever 14 in. outside the mid-line, all pulls were approximately 20% less with the oblique type, and 50% less with the alternative type of pull.

With the hand-grip at 15 in. above the seat and the arm comfortably extended, but with the subjects using a foot-rest placed in the optimum position for pedal-pushing given above, the subjects could then exert 137±1·0 and 134±1·1 lb. (20) respectively as opposed to 77·6±3·0 and 72·2±4·3 lb. (10) with the feet flat on the floor and to approximately only 30 lb. each with no foot-rest and the feet dangling. In the latter case the pull was difficult to measure as the subject tended to pull himself off the seat.

(*d*) *Maximum push at* 45° *upwards.* A hand-grip designed for any vertical direction of pull cannot be placed far above but may be placed below seat level and involves bending forward from the back-rest. Preliminary experiments showed the exertable pull in a truly vertical direction, at right angles to the floor, not to be appreciably different from that at 45°; the latter was therefore fully investigated as it permitted greater freedom of position for placing the grip.

The same procedure as before was followed and the results are given in Table 4. It will be seen that, in this case, the pull is chiefly influenced by the degree of hip-flexion possible because this determines the extent to which the powerful extensor muscles of the back can be used. Thus the height of the hand-grip in relation to the seat has a greater effect on exertable pull than distance from the back-rest, once the back is bent forward. In this case the pull decreases with hip-joint extension; this result, however, probably only represents less use of the powerful back muscles as the back approaches the seat back-rest and is thus not truly comparable with the other results.

DISCUSSION

In general the increase shown in exertable push with increase in angle of the acting joint is explained by the fact that although the length of the acting muscle decreases, and consequently also the tension developed (Evans & Hill, 1914), the mechanical disadvantage against which the muscle acts also diminishes with joint extension and more than compensates for the effect of muscle length. This has been fully considered by Franke (1920), Duboux (1942) and Haxton (1945*b*), and the results reported agree with their findings.

The above, however, does not explain the 'limiting angle' for the position of the control which allows maximum push, nor the very rapid rise in pushing force exertable with the leg (Fig. 3) and also, but to a lesser extent, with the arm (Table 2) as the acting joint approaches this angle. The explanation seems to be that, in pushing against a seat back-rest, the limb acts as a 'toggle' between the isometric control and the back-rest when the angle of the acting joint is large. The case of a pedal placed so that its bar is on the same level as the acetabulum of the subject is considered in Fig. 4.

TABLE 4. Mean maximum pull on an isometric hand-lever with direction of pull at 45° upwards, but at right angles to the lever, for grip at different distances and heights in relation to the seat. All results for the right arm with the to-and-fro plane of pull through the shoulder

Distance from centre of hand-grip to seat back (in.)	Height of centre of grip above seat level (in.)	Mean maximum pull ± 2 × S.E. (10) at 45° upwards (lb.)	
		Subject 1	Subject 2
17	− 6	80·6 ± 4·7	86·5 ± 3·5
	+ 7	—*	—*
	+13	73·4 ± 2·1	79·4 ± 1·7
	+19	—*	—*
23	− 6	98·9 ± 3·1	95·0 ± 4·8
	+ 7	—*	—*
	+13	72·8 ± 2·8	78·6 ± 2·1
	+19	58·8 ± 3·2	53·4 ± 1·3
29	− 6	102·5 ± 2·0	98·3 ± 4·8
	+ 7	95·4 ± 2·9	93·7 ± 2·6
	+13	74·3 ± 2·7	73·7 ± 1·8
	+19	53·2 ± 2·2	50·3 ± 2·7
35	− 6	102·0 ± 1·6	92·3 ± 2·9
	+ 7	77·4 ± 2·4	82·6 ± 2·5
	+13	62·7 ± 1·9	65·3 ± 1·6
	+19	47·9 ± 2·9	49·0 ± 0·75

* No readings possible with the apparatus for these positions.

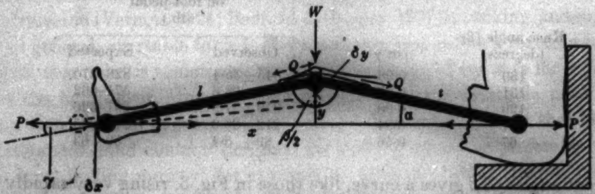

Fig. 4. Diagram showing the theoretical relationship between tension (Q) in the quadriceps muscle and force (P) exertable on an isometric pedal (placed on the same level as the acetabulum). For details, see text.

The length of the lower leg (l), from the pedal to knee-joint is considered as approximately equal to the length of the thigh (t) from knee-joint to acetabulum; this is justifiable on anthropometric grounds. The pedal is isometric and the force P exerted horizontally on it will produce an equal and opposite reaction against the back-rest.

Then, when the subject is exerting his maximum effort, the tension (Q) developed in the quadriceps, tending to increase the knee-angle (β) is equivalent to a resultant force (W) applied along a line bisecting (β), where $W=f(Q)$ depending on friction, magnitude of (β), etc.

If the knee-joint moves through a distance δy, then the pedal moves δx and work done

$$W\delta y=2P\delta x, \quad \text{or} \quad P=W\frac{\delta y}{2\delta x},$$

but

$$x=l\sin\tfrac{1}{2}\beta, \quad y=l\cos\tfrac{1}{2}\beta.$$

Therefore

$$\frac{dx}{d\frac{1}{2}\beta}=l\cos\tfrac{1}{2}\beta, \quad \frac{dy}{d\frac{1}{2}\beta}=-l\sin\tfrac{1}{2}\beta,$$

that is

$$P=W\frac{\sin\frac{1}{2}\beta}{2\cos\frac{1}{2}\beta}, \quad \text{or} \quad P=W\,\tfrac{1}{2}\tan\tfrac{1}{2}\beta.$$

Thus, the force P applied horizontally on an isometric pedal in this position will be derived from the force W, dependent on the power of the quadriceps when contracting maximally, multiplied by the tangent factor, dependent on the knee-angle β. A graphical plot of the latter factor, $\frac{1}{2}\tan\frac{1}{2}\beta$, against different angles of β gives a curve, like those in Fig. 3, rising very rapidly after about $\beta=120°$ but continuing, in this case, asymptotically to an ordinate of infinity at $\beta=180°$.

Table 5. Comparison of observed values (for maximum push exertable on an isometric pedal placed, at the limiting knee-angle, on approximately the same level as the acetabulum) with values calculated from $P=W\,\frac{1}{2}\tan\frac{1}{2}\beta$, when $W=330$ lb. (The range given for the expected values allows an estimated error of ±1° in measuring knee-angles)

Knee-angle (β) (degrees)	$\cos\gamma$	Value for maximum push on foot-pedal (lb.)	
		Observed	Expected
160	0·98	845 ± 35·4	828–970
151	0·96	684 ± 38·6	571–662
129	0·90	319 ± 27·8	297–326
117	0·84	212 ± 28·0	216–235
67	0·56	89 ± 5·4	59–63

The theory gives reasonable agreement with those experimental findings to which it can be directly applied. In Table 5, the results for maximum push on a foot-pedal given in Table 1 for a thigh-angle of 15°, i.e. when the pedal was in fact approximately on the same level as the acetabulum at the limiting angle, are compared with expected values when W is given a constant value of 330 lb.

This working is only justifiable, and the results only agree, when β is large and changes little. Under these conditions the force in the quadriceps will remain approximately constant, and the correction given to the calculated values, i.e. multiplication by cos γ (Fig. 4) as the push was actually down the line of the tibia, is small.

A value $W = 330$ lb. means a tension in the quadriceps tendon of between 700 and 950 lb. with β between 150° and the limiting knee-angle. This agrees with figures calculated by Haxton (1945*a*) and Duboux (1942).

When the pedal is below seat level the same explanation is thought to hold, but the resultant force from a push is no longer at right angles to the back-rest and the latter cannot therefore be fully used, and the subject tends to push himself up off the seat when exerting his maximum effort.

Theoretically, exertable force increases indefinitely up to a knee-angle of 180°, i.e. till the leg is mechanically straight but about 5° from being anatomically straight. Actually, as might be expected, it was found that the seat adjustment became extremely critical, and play in the leg-joints together with small movements of the pelvis determined the practical limiting angle for an isometric pedal at about 160°. It is thought that the knee-angles given by Müller (1935) for the positions allowing maximum push are too small, and the values for maximum push that he records are therefore lower, apart from any difference in experimental subjects. Otherwise the results agree well.

For the arm the same explanation holds; the results given here agree with those obtained under slightly different conditions by Franke (1920), the practical limiting elbow-angle in both cases being approximately 135°.

The similarity between the curves given here for maximum exertable force against joint extension, and between those given by other workers for living and isolated limbs that have already been quoted, and between those for hip-joint extension (Vernon, 1924; Bedford & Warner, 1937) is striking, and suggests that, in general, exertable force is at a maximum just before a reacting joint is straight; this point is sometimes overlooked in design of controls for industry.

For the hand-lever, 'toggle' effect accounts for the exertable push being greater than the pull in comparable positions. This might suggest that in some circumstances hand controls designed for pushing would be preferable to the more usual pulling variety; for example, a 'push-on' rather than a 'pull-on' hand-brake for heavy vehicles. This is fallacious. As already mentioned, pushing hard against a back-rest on a hand control was subjectively most unpleasant; moreover, if a foot-rest is provided in a position allowing limiting knee-extension, thus permitting the subject to wedge himself very firmly in his seat, possible pull becomes more than push. Alternatively the control may be placed below seat level for pulling, when the pull becomes similarly increased (Table 4).

SUMMARY

1. An account is given of experiments designed to determine how the maximum pull or push exertable on an isometric hand control, and the maximum push exertable on an isometric foot-pedal, vary with different control

positions relative to a seat. It is shown that, in general, push or pull increases with extension of the acting joint until a maximum is reached just before the joint becomes straight.

2. For pushing on a control, against a seat back-rest, the findings agree with the theory that the limb acts as a mechanical 'toggle' between the control and the back-rest. This toggle-action markedly increases the exertable push, and the relationship between control and seat becomes highly critical; it was found that the action is stopped at a well-defined 'limiting angle' and that the effect was more pronounced in the lower limb. The 'limiting angles' found were 160° for knee-extension and, approximately, 135° for elbow-extension.

3. For an isometric foot-pedal placed in front of a seat, maximum push is attainable when the subject's thigh is about 15° to the horizontal and his knee is extended just to reach the limiting angle. In this position mean maximum efforts for three different, selected, groups of subjects were 845 ± 35 lb. (6), 691 ± 66 lb. (32), and 689 ± 62 lb. (16).

4. For an isometric hand-lever, maximum pull or push is attainable when the elbow is extended up to the limiting angle, the hand-grip is about at elbow height for the seated subject, and the lever moves in a vertical plane which passes through the shoulder-joint.

5. To exert pressure between an isometric hand-grip and a seat back-rest is subjectively very unpleasant, though the exertable push, owing to toggle-action, is greater than the pull under comparable conditions. For reasons given, it is concluded that the conventional 'pull-on' hand-brakes for vehicles are preferable to a 'push-on' variety.

This work was initially done for the Military Personnel Research Committee of the Medical Research Council, to whom it was submitted as Progress Reports. The paper here includes the fundamental results from these reports, omitting the specific practical applications for which the work was undertaken.

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THE FERRICYANIDE METHOD OF BLOOD-GAS ANALYSIS

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It has long been recognized (Morawitz, 1909; Plesch, 1909; Douglas, 1910) that an error occurs in the determination of the percentage oxygen capacity of the blood by Haldane's ferricyanide method, since on adding ferricyanide to liberate the oxygen from its combination with oxyhaemoglobin a slow secondary oxidation also takes place, with the result that the estimated volume of oxygen liberated from combination is too low. In van Slyke's method such an error is inappreciable, since any oxygen set free in the reaction is instantly evacuated from the laked blood, and it has therefore been customary in recent years to regard van Slyke's method as the standard method for blood-gas analysis. On the other hand, Haldane's method has the advantage that the apparatus required is simple and cheap to construct, nor does it require the considerable quantity of mercury necessary in the van Slyke apparatus. We have therefore examined the Haldane method to see if it is possible to overcome the fallacy to which it is liable.

Parsons & Parsons (1927), Litarczek (1928), Wright & Arthur (1931) and Wright, Conant & Kamerling (1931) have shown that the error arises, at least in the main, from the oxidation of lipoid material in the blood, and is the greater in conditions of lipaemia; possibly the proteins may be concerned as well. Haldane originally mixed the blood with dilute ammonium hydroxide (2 ml. ammonium hydroxide solution, sp.gr. 0·880, later increased to 4 ml., diluted to 1 l.) or with 1% (w/v) anhydrous Na_2CO_3 in order to ensure that CO_2 should be retained in combination while the reaction between oxyhaemoglobin and potassium ferricyanide was taking place, a small quantity of saponin being added to ensure laking of the corpuscles, but the observations of Wright *et al.* as well as those of Woodhouse & Pickworth (1930) point to the fact that the speed of the secondary oxidation is dependent on the alkalinity. The latter authors found that it was possible to inhibit the secondary oxidation by working close to the neutral point, but it should be noted that in determining the percentage oxygen capacity they first thoroughly aerated the blood so that its natural CO_2 content was greatly reduced. But even if a correct estimate of the percentage oxygen capacity could be found in this way the use of the Haldane method for accurate work would still be limited, since blood-gas analysis is often needed of samples of blood containing their natural CO_2 content. It therefore seemed to us worth investigating whether it was possible to use in the Haldane method a buffer solution having a pH sufficiently low to render the secondary oxidation inappreciable and yet at the same time sufficiently high to keep the CO_2 in the blood from escaping. This could obviously not be very close to the neutral point. We first tried a borate buffer solution of pH 9·5, which showed considerable promise, but following a suggestion made to us by Dr F. J. W. Roughton we increased the pH to 10·0 and with this got very satisfactory results.

In making the buffer solutions we have followed the directions given by Walpole (1911): 'prepare a solution of "NaH_2BO_3" containing in 1 l. 12·404 g. boric acid and 100 ml. N-sodium hydrate,

> for pH 9·5 add 2 ml. N/10-NaOH to 8 ml. NaH_2BO_3 solution,
> for pH 10·0 add 4 ml. N/10-NaOH to 6 ml. NaH_2BO_3 solution,
> for pH 12·4 add 6 ml. N/10-NaOH to 4 ml. NaH_2BO_3 solution.'

In all our experiments the blood has been rendered incoagulable by the addition of 0·2% potassium oxalate and 0·1% sodium fluoride. Human blood samples were taken by venepuncture.

We have used the last form of constant-pressure blood-gas apparatus designed by Haldane (1920, cp. also Douglas & Priestley, 1937). As has been our practice for many years we have immersed the complete apparatus in a water-bath with a glass front, the upper ends of the levelling tubes projecting above the water level, and have adopted mechanical shaking of the reaction flask. The stout wire frame to which the gas burette and gauge tubes are secured is firmly held by a clamp, and the position of the liquid menisci can be accurately read without interruption of the shaking in spite of the movement of the reaction flask and rubber connecting tube. In all our experiments the water-bath has been at room temperature.

Fig. 1 shows the mechanical device for shaking the reaction flask and also shows the form of reaction flask which we have adopted. Ferricyanide solution is placed in one of the small side tubes which slopes at an angle of 45° from the lower part of the neck of the flask; tartaric acid solution can be placed in the other side tube which slopes in the opposite direction. On tilting the reaction flask appropriately, either the ferricyanide solution or the tartaric acid (used to liberate CO_2) can be run into the flask: to do this the shaking gear to which the reaction flask is attached must be lifted sufficiently from the bath to permit the requisite tilting, and then replaced. The glass tube fitted with a tap, which penetrates the rubber stopper, fits into clips on the shaking gear and also allows the air in the reaction flask, connecting tube and burette system, to be displaced by any gas mixture that may be desired.

As designed by Haldane, the reaction flask had a volume of about 20 ml., while we have used a larger flask holding 34 ml., and sometimes one holding 44 ml., which we felt might allow more effective shaking of the blood-buffer mixture. An error, which is the more significant the larger the reaction flask, can arise if the small amount of CO_2 in the normal air in the flask at the beginning of the experiment has not been completely absorbed by the buffer solution before the first reading is made, and this may require many minutes if the flask is stationary in the water-bath owing to the slowness of diffusion. To avoid this error we always blow CO_2-free air through the apparatus, introducing it by means of the glass tube shown in Fig. 1, after the reaction flask has been immersed in the water-bath before the taps are closed.

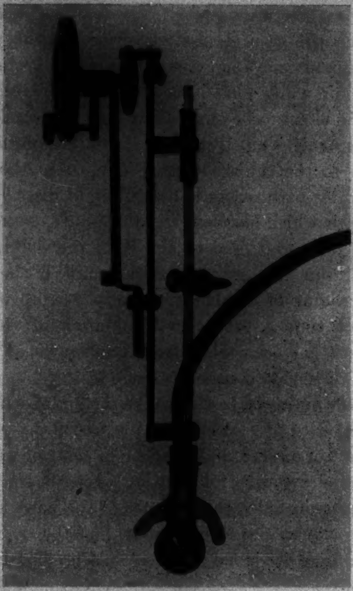

Fig. 1. Shaking gear and reaction flask used with the Haldane blood-gas apparatus.

On adding a saturated solution of potassium ferricyanide to water or to a mixture of the buffer solution and blood plasma, we have found that a small diminution of volume is registered in the gas burette, possibly because of a change of vapour pressure. A correction can be applied for this change in volume, but it is simpler to substitute a 6% (w/v) solution of potassium ferricyanide for the saturated solution, as with this the change in volume is inappreciable; 0·25 ml. of this 6% solution is ample to expel the oxygen from 2 ml. of normal blood, even though a little of the solution is unavoidably left in the side tube on tilting the reaction flask. The dilute solution has, too, the advantage over the more concentrated, since the rate of the secondary oxidation is considerably diminished.

Our procedure has therefore been as follows: 1·5 ml. borate buffer solution of pH 10·0 (or such pH as is desired) is placed in the reaction flask, and 0·25 ml. 6% potassium ferricyanide solution, in which a small pinch of saponin (the so-called 'saponin white') has been dissolved, is placed in one of the side tubes; 1·0 ml. of the blood to be analysed is then run carefully from a pipette (Ostwald or narrow straight pipette) under the buffer solution and the reaction flask is pushed on to the rubber stopper, clipped to the shaking gear and immersed in the water-bath. Care is taken not to disturb the two layers of blood and buffer. Air which has been freed from CO_2 is then run through the apparatus and the taps are closed to air. With the shaking gear stationary, temperature equilibrium is reached in 2 or 3 min. if the bath is efficiently stirred; a steady reading of the burette is obtained up to the 10th or 15th minute, after which time a very slow diminution of volume may become apparent if the blood has been taken from a saturator at 38° C., as in determining the oxygen-dissociation curve, because of the slow diffusion of oxygen and nitrogen from the air in the flask through the buffer solution into the blood which has now cooled to the lower temperature of the water-bath and can hold a larger amount of oxygen and nitrogen in solution and combination. If the blood has been previously fully saturated with air at about the same temperature as the water-bath, the readings of the burette remain steady for many minutes. An example of this is shown in Table 1. The temperature of the water-bath was 16·5° C.; blood sample A had previously been saturated at

TABLE 1. Readings in ml. of gas burette after closing taps with the reaction flask stationary. To show the influence of diffusion of gases through the buffer solution into the blood

Min. after closing taps	Blood sample A	Blood sample B
2	0·153	—
3	0·151	0·182
4	0·151	0·181
5	0·150	0·180
6	0·150	0·180
8	0·150	0·180
10	0·150	0·180
12	0·150	0·180
14	0·150	0·180
19	0·149	0·180
22	0·148	0·180
25	—	0·180

38° C. with air containing 5·52% CO_2, 6·90% O_2 and 87·58% N_2, while sample B had previously been saturated at 16·5° C. with normal air.

When the initial steady reading has been verified the shaking is started, and any oxygen uptake is noted when the readings have been steady for 5 min.; we have found no change in volume if the blood has previously been thoroughly saturated with normal air at room temperature. The ferricyanide solution is then run into the reaction flask and shaking continued until a steady reading shows that the combined oxygen has been set free from the laked blood.

That the borate buffer of pH 10·0 can prevent any significant escape of CO_2 from the blood while one of pH 9·5 is hardly adequate is shown in Table 2.

TABLE 2. Change in gas volume in ml. on shaking blood with buffer solutions. Blood saturated at room temperature with air or air + CO_2, and measurement of volume made at the same temperature

Borate pH 9·2		Borate pH 9·5		Borate pH 10·0	
CO_2 content ml./100 ml. blood	Change on shaking	CO_2 content ml./100 ml. blood	Change on shaking	CO_2 content ml./100 ml. blood	Change on shaking
60·5	+0·009	54·0	+0·002	61·4	0·000
60·0	+0·007	63·9	+0·004	62·5	0·000
		67·6	+0·003	63·1	0·000
				67·1	−0·001
		Low CO_2 content	0·000	67·6	0·000
			+0·001	68·2	0·000
			+0·001	71·0	0·000

Blood was saturated with oxygen at room temperature by exposure in a saturator either to normal air, which also greatly reduced the CO_2 content of the blood, or to air to which 2·4–4·3% of CO_2 had been added, in which case the total CO_2 content of the blood reached a high value. The blood was then placed under the buffer solution in the reaction flask in the usual way, and after the initial steady reading had been noted shaking was started. Whereas no further change in volume occurred with the buffer of pH 9·5 when the CO_2 content of the blood was low, when the CO_2 content was high there was a distinct, though slight, *increase* in volume with the buffer of pH 9·5 and a still greater increase with a buffer of pH 9·2 ('NaH_2BO_3' solution without addition of sodium hydroxide), although the volume remained unchanged with the buffer of pH 10·0, suggesting that at the lower pH some CO_2 passed into the gaseous phase.

Fig. 2 shows the degree to which the borate buffer of pH 10·0 inhibits the secondary oxidation in blood and plasma. The upper two graphs were obtained from a single specimen of human blood and the plasma separated from it; the lower two graphs give the mean results of experiments on the blood and plasma of each of two rabbits. The blood or plasma was put under the buffer or sodium carbonate solution and shaking started. When equilibrium had been reached the ferricyanide was run in. Since the plasma was practically free from haemoglobin, readings of the diminution of volume due to the secondary oxidation could be started at once. In the case of blood, oxygen had first to be expelled from combination, and the graphs therefore start when a maximum reading was first reached 4–5 min. after running in the ferricyanide.

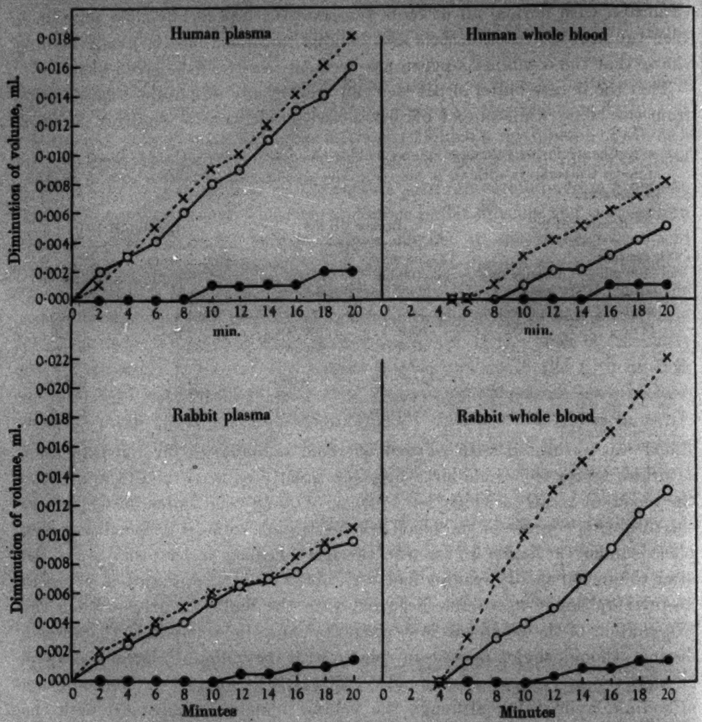

Fig. 2. Rate of secondary oxidation after adding 0·25 ml. 6% potassium ferricyanide to 1·0 ml. plasma or whole blood and 1·5 ml. buffer or sodium carbonate solution. ● Borate buffer pH 10·0. ○ Borate buffer pH 12·4. × 1% Na_2CO_3.

Using a borate buffer of pH 10·0 the initial reading remains steady for some minutes, after which the volume begins to show a just perceptible diminution. It seems clear, therefore, that with the buffer of pH 10·0 the secondary oxidation is so slow that it should not cause any appreciable error in the measurement of the oxygen liberated from combination with haemoglobin.

This is certainly not the case when a borate buffer of pH 12·4 or 1% Na_2CO_3 is used. The rate of secondary oxidation is now so much increased that it is clear that it must influence the reading while oxygen is being liberated and make the estimate of the oxygen combined with haemoglobin too low. Ox blood and plasma behave very much like human blood, the rate of secondary oxidation being faster in plasma than in whole blood, but in the blood of several rabbits which we have tested the rate of oxidation was faster in the blood than in the plasma separated from it. The rate of the secondary oxidation varies with blood from different species, and with blood from different individuals of the same species; it is particularly troublesome in rabbit's blood, but even in this case Fig. 2 shows that the use of a borate buffer of pH 10·0 ought to render it negligible.

The percentage oxygen capacity of samples of blood which had been thoroughly aerated at room temperature with normal air, to which in some cases CO_2 was added so as to maintain a high concentration of CO_2 in the blood, was determined by both the van Slyke method and by the Haldane method, using in the latter case borate buffer solutions of pH 9·5, 10·0 and 12·4 or a 1% solution of Na_2CO_3. In calculating the results by the van Slyke method the coefficients of solubility of oxygen given by Sendroy, Dillon & van Slyke (1934) have been used. The results are given in Table 3.

TABLE 3. O_2 capacity of whole blood, ml. O_2/100 ml. blood, determined by van Slyke and Haldane methods

		Haldane			
	van Slyke	Borate pH 9·5	Borate pH 10·0	Borate pH 12·4	1% Na_2CO_3
Human	18·3	18·5	—	18·1	17·9
	18·4	18·4	—	18·0	17·6
	19·6	19·7	—	19·0	18·8
	17·6	—	17·8	17·4	17·0
	20·4	—	20·5	20·0	19·6
	18·8	—	18·8	18·4	17·6
	18·3	—	18·2	17·7	17·6
	15·4	—	15·4	14·9	14·7
	20·5	—	20·5	—	—
	15·8*	—	15·6*	—	—
Rabbit	12·8	12·8	—	12·4	11·9
	14·1	14·1	—	13·6	13·3
	12·8	—	12·9	12·4	11·5
	14·4	—	14·3	14·0	13·5
Sheep	16·6	—	16·7	16·2	15·9
	17·1†	—	17·1†	—	—
Ox	20·3	—	20·4	19·9	19·5
	21·8	—	21·8	21·4	21·5
	21·5	—	21·5	21·2	21·1
	21·4	—	21·4	—	—
	21·8‡	—	21·7‡	—	—

* 61·4 ml. CO_2/100 ml. blood.
† 67·6 ml. CO_2/100 ml. blood.
‡ 71·0 ml. CO_2/100 ml. blood.

Whereas the Haldane method with the borate buffer of pH 12·4 gives results which are lower in every case than those given by the van Slyke method, and lower still when Na_2CO_3 solution is used (which is in agreement with the results recorded by others who have used Na_2CO_3 or NH_4OH solutions, e.g. van Slyke & Stadie, 1921; Price-Jones, Dill & Wright, 1931; Bierring, Nielsen & Nielsen, 1936; Douglas, Jope, Jope, Macfarlane & O'Brien, 1943), with the borate buffer of pH 9·5 when a low CO_2 content is present in the blood, and with one of pH 10·0 even when the CO_2 content of the blood is high, the results obtained by both the Haldane method and the van Slyke method are practically identical. A borate buffer of pH 10·0 seems therefore to fulfil the required conditions.

As a further test of the validity of the Haldane method with the modifications which we have introduced we have determined the oxyhaemoglobin dissociation curve in the blood of three different individuals. The blood was equilibrated at 38° C. by the method described in detail by Douglas & Priestley (1937) with mixtures of nitrogen and different concentrations of oxygen, CO_2 being added in each instance so as to afford a partial pressure of this gas of about 40 mm. Hg. To obtain the lowest concentrations of oxygen the blood was first brought into equilibrium with nitrogen at 38° C. so that most of the oxygen was removed from it; the saturator was then refilled with nitrogen containing the required amount of CO_2 and equilibration completed. The percentage saturation of the haemoglobin with oxygen was determined independently by C. G. D. using the Haldane method with borate buffer of pH 10·0 and by F. C. C. using the van Slyke method. The results are given in Table 4.

With the gas burette which we have used in the Haldane apparatus we cannot detect a change in volume of less than 0·001 ml., corresponding to an alteration in volume of 0·1 ml./100 ml. blood when using 1·0 ml. blood for analysis. When calculating the percentage saturation of haemoglobin with oxygen this means that differences of less than 0·5 are certainly without significance. We doubt whether in our hands the van Slyke apparatus gives results of any greater accuracy than this when using 1 ml. blood if these have to be based on single determinations, as is the case in the figures shown in Table 4. We have therefore expressed the percentage oxygen saturation to the nearest 0·5. In the Haldane method the amount of oxygen taken up to complete saturation of the haemoglobin, and the amount subsequently expelled by ferricyanide, are determined in the same sample of blood, and a small error in the volume of blood delivered from the pipette has no significance. The van Slyke method involves the measurement of the actual oxygen content in two separate samples of blood of known volume, and there is the possibility of a small error arising in the delivery of blood from the pipette. In calculating the results by the van Slyke method we have in each instance taken for the percentage oxygen capacity of the fully oxygenated blood the figure given by the Haldane method on the same sample of blood, a procedure which we feel is justified by the results given in Table 3.

TABLE 4. Oxyhaemoglobin dissociation curve of human blood determined by van Slyke and Haldane methods

		% saturation of Hb with O_2		
			Haldane	
Subject	O_2 tension mm. Hg	Van Slyke	Bohr coeff.	van Slyke coeff.
C. G. D.	1·4	1·5	1·5	2·0
	9·9	10·0	10·0	10·5
	17·7	24·0	24·0	25·0
	20·4	30·5	31·5	32·0
	30·0	52·0	53·0	53·5
	40·8	73·0	73·0	74·0
	48·3	83·5	83·0	83·5
	60·3	91·5	91·0	91·5
	79·2	95·0	95·0	96·0
	98·1	96·5	97·0	97·5
F. C. C.	1·0	1·0	1·5	2·0
	8·5	8·5	10·0	10·0
	11·6	14·5	13·5	14·0
	18·1	30·5	30·0	30·5
	28·1	53·5	54·0	54·5
	37·6	72·5	73·5	74·5
	49·0	86·5	86·0	86·5
	59·5	91·5	93·0	93·5
	79·3	97·0	95·5	96·0
	101·9	98·0	97·5	98·0
T. J. M.	0·9	1·0	0·5	1·0
	11·2	12·0	11·5	12·0
	16·6	21·0	20·0	20·5
	22·8	38·0	37·0	37·5
	30·6	56·0	56·0	56·5
	40·1	72·0	73·5	74·0
	49·0	83·5	84·0	84·5
	63·6	90·5	91·5	92·0
	80·5	96·5	95·0	95·5
	100·4	99·0	97·0	98·0

The results obtained by the Haldane method have been calculated (*a*) using the Bohr coefficients of solubility of nitrogen and oxygen in blood since these have been used by many observers in the past, and (*b*) using the more recent coefficients determined by van Slyke, Dillon & Margaria (1934) and Sendroy *et al.* (1934). In the latter case we have used for oxygen the coefficients of solubility of 0·023 at 38° C. and 0·038 at 15° C., and for nitrogen 0·013 at 38° C., but in the absence of exact data at lower temperatures we have assumed a coefficient of 0·018 at 15° C., and this figure may not be quite correct. The substitution of the van Slyke for the Bohr coefficients increases the apparent percentage saturation with oxygen by about 0·5 on the average, but, whichever figures are taken, agreement between the results obtained both by the Haldane method and by the van Slyke method is extremely close. Out of the total of thirty observations the percentage saturation with oxygen determined by the Haldane method only differs from that determined by the van Slyke method by more than 1·0 in seven instances using the van Slyke coefficients, and in six instances using the Bohr coefficients. The close agreement between the results obtained by the two methods is clearly seen in Fig. 3, which shows the dissociation curve drawn for the blood of C. G. D. from the data in Table 4 which were obtained by the van Slyke method and by the Haldane method using the van Slyke coefficients of solubility. We feel therefore that, with the modifications which we have described, the Haldane method will yield results for the percentage saturation of haemoglobin in the blood which are to all intents and purposes identical with those given by the van Slyke method.

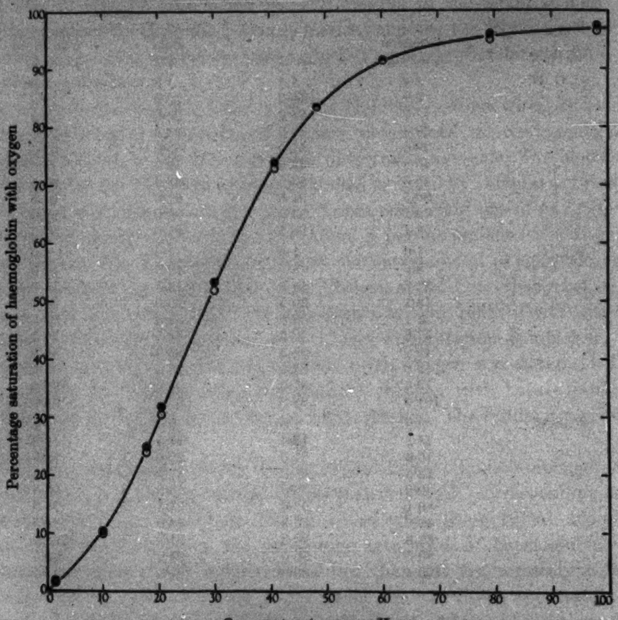

Fig. 3. Oxyhaemoglobin dissociation curve of blood of C. G. D.
● Haldane method. ○ van Slyke method.

The values given by the Haldane method should be a little more accurate if 2 ml. blood were used, as Haldane originally recommended, since the volume of gas to be measured would be doubled while the minimal detectable alteration in volume would be unchanged, but our results show that adequate accuracy can be secured using only 1 ml. blood provided that the readings are made with care. The smaller volume of blood has, too, the advantage that a shorter time is required for oxygen saturation and subsequent oxygen liberation, and, when required, the CO_2 content can afterwards be determined in the same sample of blood.

Only normal blood has been used in our experiments: we have had no opportunity for testing lipaemic blood in which the secondary oxidation on adding ferricyanide is more rapid, although we made quite satisfactory determinations of the percentage oxygen capacity of samples of normal rabbit's blood in which the rate of secondary oxidation on adding ferricyanide was far quicker than in normal human blood.

SUMMARY

1. In the Haldane ferricyanide method of blood-gas analysis an error in the measurement of the oxygen content of the haemoglobin is caused by a secondary oxidation due to the action of ferricyanide on lipoid substances and perhaps proteins in the blood.

2. This error can be eliminated by the substitution of a borate buffer of pH 10·0 for the sodium carbonate or ammonia solution which has usually been employed, and the substitution of a 6% solution of potassium ferricyanide for a saturated solution.

3. A further source of error can be avoided by running CO_2-free air through the apparatus before closing the taps.

4. With this modified procedure the Haldane method has been found to give results which are to all intents and purposes identical with those given by the van Slyke method. This has been confirmed by determinations by the two methods of the percentage oxygen capacity of fully oxygenated blood and the haemoglobin dissociation curve.

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EXPERIMENTS ON LIVER SYMPATHIN

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The properties of the substance liberated on stimulation of the adrenergic nerves to an isolated frog's heart or a perfused rabbit's ear have been much studied and there can be little doubt that this substance is adrenaline (Loewi, 1936; Gaddum & Kwiatkowski, 1938, 1939; Gaddum, Jang & Kwiatkowski, 1939). On the other hand, it is difficult to account for all the facts on the theory that the action of adrenergic nerves is entirely due to the liberation of adrenaline. Thus Barger & Dale (1910) concluded that this theory 'involves the assumption of a stricter parallelism between the two actions than actually exists. Adrenine has, in common with other methylamino-bases of its catechol group, the property of exaggerating inhibitor as compared with motor effects. ...The action of some of the other bases, particularly of the amino- and ethylamino-bases of the catechol group, corresponds more closely with that of sympathetic nerves than does that of adrenine'.

Cannon, working with various collaborators, obtained evidence that stimulation of sympathetic nerves in a cat with normal circulation produces on other organs effects attributed to the liberation into the blood-stream of two substances, sympathin E, with mainly excitor actions and sympathin I, with mainly inhibitor actions. This conclusion has been upheld by later work from the same laboratory. The evidence has been summarized by Cannon & Rosenblueth (1937), and the results have been confirmed in other laboratories, but the chemical nature of sympathins E and I is still speculative.

The evidence which is most clearly incompatible with the view that adrenaline is the only substance liberated was obtained by stimulating the hepatic nerves of cats, which caused effects attributed to the liberation of sympathin E, uncontaminated with sympathin I. The stimulation of other nerves caused effects attributed to both sympathins. There is no evidence that sympathin I is ever liberated uncontaminated with sympathin E. This gap in the evidence may be due to the fact that the motor nerves to the blood vessels in all the organs which have been studied liberate sympathin E which thus cannot be separated from the sympathin I liberated by the organs themselves (Cannon & Rosenblueth, 1937). On the other hand, the results can be explained on the theory that sympathin I is adrenaline. Bacq (1935) suggested this possibility, but later work led him to a more attractive theory, since he found that various extracts containing catechol oxidase converted adrenaline, under appropriate conditions, to a vasodilator substance (adrenoxine). He suggested that adrenaline inhibition was due to the presence of a catecholoxidase in the inhibited structures (Bacq, 1938; Heirman & Bacq, 1940).

The experiments described here were undertaken to study the properties of the substance liberated on stimulation of the hepatic nerves in the cat. This substance, or mixture of substances, will be called liver sympathin. Its known properties are similar to those of noradrenaline which is identical with adrenaline, except for the absence of the *N*-methyl group; it was studied by Barger & Dale (1910) and found to have excitor actions about equal to those of adrenaline, but little or no inhibitory action. The theory that liver sympathin is noradrenaline or some similar substance was advanced by Bacq (1933) and by Stehle & Ellsworth (1937). Greer, Pinkston, Baxter & Brannon (1938) gave a full discussion of the question and supported the theory with direct comparisons between the actions of noradrenaline and liver sympathin, but it was thought desirable to amplify the evidence.

We have devoted particular consideration to one possible explanation of the results, in addition to those discussed above. In experiments of this type the adrenaline is usually injected rapidly, but the nerves may perhaps continue to liberate sympathin for at least as long as they are stimulated. By comparing quick injections of adrenaline with slow injections, it is possible to obtain discrepancies similar to some of those observed by Cannon & Rosenblueth, and it is therefore necessary to consider the theory that all the effects are due to the slow liberation of adrenaline by the liver.

METHODS

In different experiments spinal cats and decerebrate cats were used and also cats anaesthetized with ether, chloralose or dial compound (allobarbitone B.P.C. 0·1 g., ethylurethane 0·4 g., monoethylurea 0·4 g./c.c.; 0·85 c.c./kg.). When spinal or decerebrate cats were used it was found best to do most of the dissection under ether before destroying the brain.

Arterial blood pressure was recorded with a mercury manometer. Contractions of the nictitating membrane were recorded isotonically. In some cases the membrane was denervated by removal of the superior cervical ganglion in a preliminary aseptic operation under general anaesthesia 7–10 days before the main experiment. The diameter of the pupil was measured with dividers. The virgin uterus was fixed at its lower end, and the upper end of one horn was freed and its movements recorded by a device similar to a Cushny (1910) myograph, while the abdomen was filled with warm NaCl solution. Movements of the duodenum were recorded by tying off a segment which was filled with warm saline and connected by a cannula and rubber tubing to a bottle, the upper part of which contained air and was connected to a piston recorder.

Most of the slow intravenous injections were given by a special device which provided a record of the rate of injection. An ordinary mercury manometer was connected by rubber tubes to a venous cannula, and also to a reservoir containing a solution of a drug in saline. Both tubes were closed with clips. When one clip was opened the mercury was depressed, and the apparatus filled at high pressure. When the other clip was released the fluid was driven into the vein by the weight of the mercury and rate of fall of the column of mercury was recorded in the usual way. The rate of flow was controlled by a brass pin-valve through which the fluid flowed between the manometer and the cannula.

The hepatic nerves were separated from the hepatic artery and divided centrally. The portal vein was cleaned, and the duodenal artery, the nerves and other tissues running down to the intestines were divided between ligatures. The bile duct was cannulated and divided. The hepatic nerves were stimulated through platinum electrodes, using either an ordinary coil, or the charge and discharge of a condenser connected with a rotary key so that alternate shocks had opposite sign and polarization was minimized. A potential of 30–50 V. passed through 1000 Ω to a capacity of 2 mF. Shocks were applied at about 40/sec. for 15–30 sec. by opening a short-circuit key. The drugs used were *l*-adrenaline HCl, *dl*-noradrenaline HCl, tyramine HCl, cocaine HCl, and histamine acid phosphate. The doses of histamine are given in terms of histamine base; the other drugs as salts.

RESULTS

Many of the results have been obtained under dial anaesthesia after a large dose of cocaine (8 mg./kg.), as recommended by Cannon & Rosenblueth. These particular conditions give good results, but are not essential to the phenomena. Stimulation of the hepatic nerves caused a rise of blood pressure, contraction of the denervated nictitating membrane and inhibition of the intestine in a spinal cat after cocaine. Cocaine is not essential, since stimulation of the nerves caused a rise of blood pressure, contraction of denervated nictitating membranes and inhibition of the intestine before cocaine had been given. However, cocaine did cause a definite increase of the effect on the nictitating membrane, and sometimes also of the effect on blood pressure.

Blood pressure

Stimulation of the hepatic nerves almost invariably caused a rise of blood pressure. Part of this effect must have been due to constriction of the hepatic artery itself, since the pressure started to rise in a few sec., before any substance from the liver could have reached the general circulation.

Clamping the hepatic artery. In order to determine whether the observed effects were entirely due to this cause, the hepatic artery was clamped. This raised the blood pressure by 15–30 mm. Hg, and the rise was maintained until the clamp was removed. This effect was unexpectedly large; clamping one femoral artery caused smaller and more transitory rises of blood pressure. Part of the effect of the clamp may have been due to the release of blood from the portal circulation into the systemic circulation, since it is known that clamping the hepatic artery causes a fall of pressure in the portal vein (McMichael, 1932). The pressor effect of stimulation cannot, however, be entirely due to this cause, because it was sometimes much larger than 30 mm. Hg, and because it was observed after the hepatic artery had been clamped and at a time when the blood pressure had reached a steady increased level after the application of the clamp. In the experiment shown in Fig. 1 stimulation of the hepatic nerves raised the blood pressure by 85 mm. Hg. Eight min. later a clamp on the artery raised it by 15 mm. Hg. Two min. later stimulation of the hepatic nerves raised the blood pressure to about the same height as when the circulation was free. This second effect cannot have been due to constriction of the hepatic artery which was already obstructed completely. It will be seen that there is a small notch on the ascending part of the first curve; this was attributed to the fact that the effect was partly due to constriction of the artery and partly due to the liberation of sympathin. This experiment shows clearly that the effect is not all due to constriction of the artery, but is not typical, since the effect of stimulation was usually less than

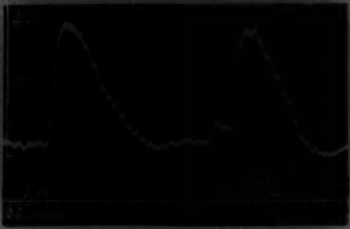

Fig. 1. Cat. Dial. Cocaine. Blood pressure. *S*, stimulation of hepatic nerves. Hepatic artery clamped at mark.

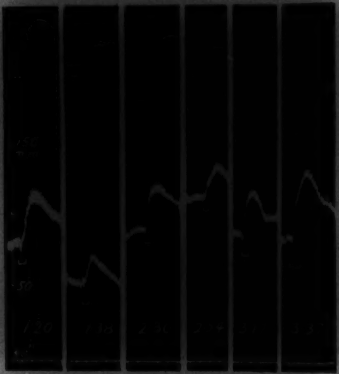

Fig. 2. Cat. Dial. Cocaine. Denervated nictitating membrane. Blood pressure. Stimulation of hepatic nerves for 15 sec. Last stimulation 30 sec. Mesenteric arteries and portal vein closed 1.26–2.00. Hepatic artery closed 2.39–3.13.

this, and the effect of clamping was usually larger. When the artery was clamped in another experiment (Fig. 2, 2.39–3.13) the blood pressure promptly rose by 25 mm. Hg, and the rise was maintained until the clamp was removed 34 min. later, when the blood pressure promptly fell to its initial level. Stimulation of the nerves during this period caused an additional rise of 25 mm. Hg. The fact that the rise of blood pressure is due to at least two causes makes difficult any quantitative comparisons between pressor effects and other effects, but a number of such comparisons was made before this source of error was fully appreciated.

Clamping the portal vein. The effects of cutting off the portal circulation were studied in the hope of localizing the site of liberation of the sympathin, and to see whether it would be necessary to maintain a double circulation if the liver were perfused to study these effects. In order to eliminate, as far as possible, the accumulation of blood in the intestines, clamps were applied as nearly as possible simultaneously to the superior and inferior mesenteric arteries and the portal vein. This caused a fall of blood pressure (Fig. 2, 1.26), and the pressor effect of stimulation progressively diminished during the 34 min. that the clamps were left in position. When the clamps were removed the blood pressure recovered in a few minutes, and the pressor effect of stimulation also recovered almost to its former value. These results and those on the nictitating membrane are discussed below.

The effect of ergotoxine. Cannon & Rosenblueth found that, after the administration of large doses of both ergotoxine (5 mg./kg.) and cocaine to the same cat, the injection of adrenaline caused a pure fall of blood pressure but that stimulation of the hepatic nerves caused a rise, and the stimulation of other sympathetic nerves caused mixed effects. We have confirmed their observations with regard to the hepatic nerves, but have come to the conclusion that they cannot be regarded as rigid proof that liver sympathin is not adrenaline. In cats which had received dial and ergotoxine, large doses of adrenaline (50 μg.) injected rapidly usually caused a pure fall of blood pressure, but after smaller doses (10 μg.) there was a secondary rise of blood pressure. When the injection was given slowly, the initial fall was less marked, and it was sometimes possible, by modifying the dose of adrenaline and the rate of injection, to imitate the pressor effect of stimulating the hepatic nerves, even when rapid injections of large doses of adrenaline caused a pure fall of blood pressure. Stimulation of the nerves, however, never caused a fall of blood pressure and it was not always possible to produce a pure rise of blood pressure with adrenaline. These facts support the view that the pressor effect of stimulation after ergotoxine is not due to the slow liberation of adrenaline into the general circulation.

There is, however, still another possible explanation of this effect. It might be due to stimulation of the hepatic nerves producing, after ergotoxine, a dilatation of the portal branches in the liver, thus releasing blood from the portal circulation into the general circulation. This theory is supported by the fact that the pressor effect after ergotoxine has a longer latent period (30–45 sec.) than before (5–10 sec.). We have observed this delay, but do not reproduce a tracing of it since it is well shown in Fig. 2 of the paper by Cannon & Rosenblueth (1933).

These observations do not disprove the theory of Cannon & Rosenblueth, but they provide alternative theories.

Effect of ether. In cats under ether, small doses of adrenaline cause a fall of blood pressure. If liver sympathin was adrenaline, appropriate stimulation of the hepatic nerves in such cats might cause a fall of blood pressure, but in our experiments it always caused a pure rise of blood pressure. This fact supports the theory that liver sympathin is not adrenaline, but it must be remembered that an initial depressor effect of stimulation might be obscured by the direct effect of constriction of the hepatic artery.

Pupil

Cannon & Uridil (1921) found that liver sympathin did not dilate the pupil, although doses of adrenaline with an equivalent effect on the heart and blood pressure did dilate the pupil. We have compared effects on the pupil and blood pressure and got similar results. Fig. 3 shows an experiment in which stimulation of the hepatic nerves caused a rise of blood pressure, but had no effect on the pupil, whereas a dose of adrenaline with a smaller pressor action did dilate the pupil widely. Results of this kind are striking, but their value

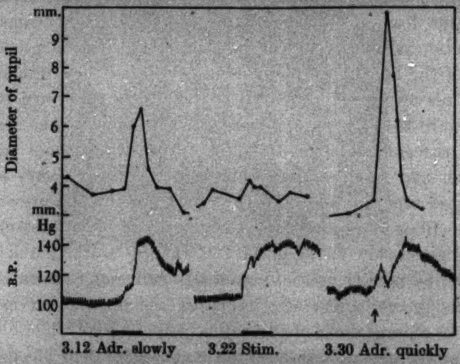

Fig. 3. Cat. Dial. Cocaine. Diameter of pupil. Blood pressure. 3.12, adrenaline 2 μg. in 1 min. 3.22, stimulation hepatic nerves for 1 min. 3.30, adrenaline 2 μg. quickly. Slow injection diminishes effect on pupil but not on blood pressure.

as evidence is lessened by the fact that diminishing the rate of administration of adrenaline may diminish its effect on the pupil and increase its effect on the blood pressure. Such a result is also shown in Fig. 3. This suggests that some of the discrepancies observed may be due to the fact that liver sympathin is liberated slowly. However, the facts that stimulation of the hepatic nerves never had a definite effect on the pupil, and that it was difficult to avoid such effects with adrenaline, does suggest that liver sympathin is not adrenaline.

Greer *et al.* (1938) found that noradrenaline resembled liver sympathin in having only a small dilator effect on the pupil, compared with that of adrenaline in equipressor doses. This observation could not have been predicted with confidence, since it is generally thought that the dilatation of the pupil by adrenaline is due to stimulation of radial muscles and should therefore be equally well produced by noradrenaline, but Greer *et al.* quote evidence that inhibition of circular muscles also plays a part in this dilatation. In any case the resemblance between liver sympathin and noradrenaline in this respect supports the view that they are identical. In one experiment we compared the effects of liver sympathin on the denervated pupil with those of equipressor doses of adrenaline, noradrenaline and tyramine. In confirmation of the results of Greer *et al.* noradrenaline had less effect than adrenaline, but tyramine had no effect at all and in this respect it resembled liver sympathin. It is known that tyramine may cause dilatation of the normal pupil, but not the denervated pupil, of a cat (Burn & Tainter, 1931).

Nictitating membrane

Cannon & Rosenblueth found that the nictitating membrane was very sensitive to liver sympathin, and that the effect was increased by degenerative section of the sympathetic supply to the membrane, or by cocaine, and inhibited by ergotoxine. These observations have been confirmed.

When either the hepatic artery or the portal vein was clamped the tone of the nictitating membrane usually increased slowly, and the effect upon it of stimulating the hepatic nerves diminished slowly. There was no sudden disappearance of the effect of stimulation, such as might have been expected, if the effect was due to a substance liberated in parts of the circulation which only received blood from one of these vessels. In the experiment shown in Fig. 2 the response of the membrane recovered after the occlusion of the portal vein; after occlusion of the hepatic artery, later in the experiment, it did not recover completely, but it still responded well when the duration of stimulation was increased. The effects of those occlusions on the nictitating membrane were similar to those on the blood pressure. They provide no evidence of the site of liberation and suggest that in experiments on perfused livers both vessels should be perfused.

In considering the substances which might be responsible for effects on the nictitating membrane it must be borne in mind that this tissue is sensitive to many drugs. Rosenblueth (1932) showed that it was not only sensitive to adrenaline, but also to acetylcholine, pilocarpine, eserine and histamine and that the effects of all these drugs were increased by previous denervation or cocaine. Ross (1936) showed that the effects of adrenaline, acetylcholine, $CaCl_2$ and KCl on the denervated membrane after cocaine were antagonized by 933F or yohimbine—both of which have been thought to act specifically against adrenaline. Both observers used cats under dial after ligation or removal of the adrenals, so that the effects were not secondary to the liberation of adrenaline from the adrenals.

We have extended the work with histamine to which the membrane was usually about as sensitive as it was to adrenaline.

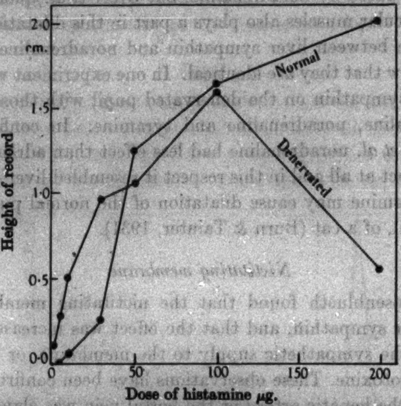

Fig. 4. Cat. Dial. Cocaine. Adrenals removed. Contraction of nictitating membranes. Intravenous injection of histamine. Denervation sensitizes to small doses but not to large doses.

In Fig. 4 the effects of various doses of histamine on the two nictitating membranes are plotted against the dose. Denervation had sensitized the membrane to small doses, but when the dose was increased the difference between the two effects disappeared and even became reversed for a very large dose. Bülbring & Burn (1938) obtained similar results with tyramine, and even in some cases with adrenaline. The fact that 200 μg. of histamine actually had less effect on the normal membrane than 100 μg. was probably due to the large fall of blood pressure caused by the larger dose. It might be suggested that this effect of histamine was secondary to the liberation of adrenaline from the adrenals (Dale, 1920; Kellaway & Cowell, 1923). These glands had however been removed before the results shown in Fig. 4 were obtained. The membranes both gave slightly smaller responses after this removal than before, but this change appeared to be part of a continuous slow loss of sensitivity, and the membrane remained very sensitive to histamine after adrenalectomy. It was clear that this effect of histamine was not caused to any significant extent by the liberation of adrenaline by the adrenals. It would be difficult to distinguish effects due to histamine from those due to adrenaline by the study of the nictitating membrane alone.

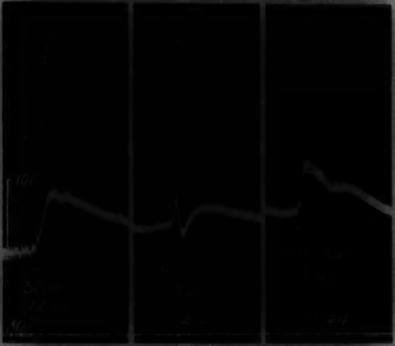

Fig. 5. Cat. Dial. Cocaine. Denervated nictitating membrane and blood pressure. Stimulation of hepatic nerves for 15 sec. Intravenous injection of adrenaline and noradrenaline.

The pressor effect of liver sympathin was always greater than that of a dose of adrenaline which had an equal effect on the nictitating membrane. This is shown in Fig. 5 from which it will also be seen that noradrenaline provided a much better imitation of the effects of nervous stimulation than did adrenaline itself.

Virgin uterus

One of the most striking pieces of evidence advanced by Cannon & Rosenblueth was the fact that liver sympathin had no effect on the virgin uterus, though doses of adrenaline with equivalent effects on other organs caused inhibition. We have confirmed the fact that liver sympathin has comparatively little effect on the uterus, but have on several occasions seen it cause inhibition, thus confirming an observation by Greer *et al.* (1938). In the experiment shown in Fig. 6 it caused slight inhibition at first (upper tracings), but later this effect disappeared and results similar to those of Cannon & Rosenblueth were obtained. At this stage (lower tracings) adrenaline inhibited the uterus and stimulated the nictitating membrane, whereas stimulation of the hepatic nerves had no action on the uterus, although it had a larger action than adrenaline on the membrane. These results confirm the evidence that liver sympathin is not adrenaline.

The possibility was considered that the difference of effects was due to the slow liberation of adrenaline in the liver compared with its rapid intravenous injection. Rapid injections of adrenaline caused prolonged inhibition of the uterus sometimes preceded by a brief contraction. When the injection was made slowly, the inhibition disappeared, but the initial contraction was sometimes increased. This diminishes the value of Cannon & Rosenblueth's evidence, but we have no direct proof that tracings like Fig. 6 can be produced by slow injections of adrenaline.

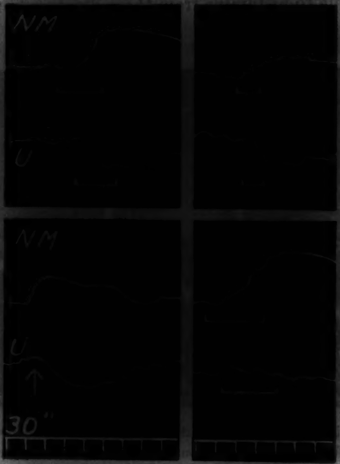

Fig. 6. Cat. Dial. Cocaine. Denervated nictitating membrane and non-pregnant uterus. Stimulation of hepatic nerves for 60, 30 and 90 sec. causes inhibition of uterus which disappears later. Adrenaline (0·5 μg.) at arrow. Inhibition of uterus and comparatively small effect on membrane.

It is possible that the small effect of liver sympathin on the uterus is due to the fact that it consists partly of histamine, which has usually caused contraction of the uterus even when adrenaline caused relaxation, but may sometimes also cause relaxation. Noradrenaline also has less inhibitory effect on the uterus than adrenaline and may stimulate uteri which are inhibited by adrenaline.

Intestine

Greer *et al.* (1938) showed that liver sympathin caused inhibition of the intestine, and that noradrenaline also inhibited the intestine, but that its effect was less than that of adrenaline. Fig. 7 shows these phenomena. In this and in other experiments the effects on the blood pressure and intestine were compared, and the results appeared to indicate that liver sympathin resembled

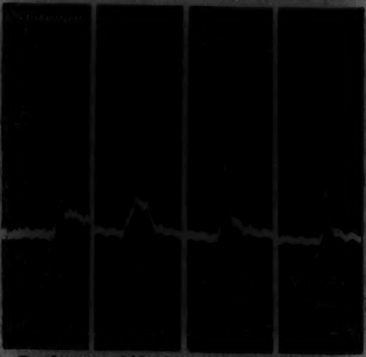

Fig. 7. Decerebrate cat. Duodenum and blood pressure. Stimulation of hepatic nerves for 1 min. Adrenaline and noradrenaline, intravenous.

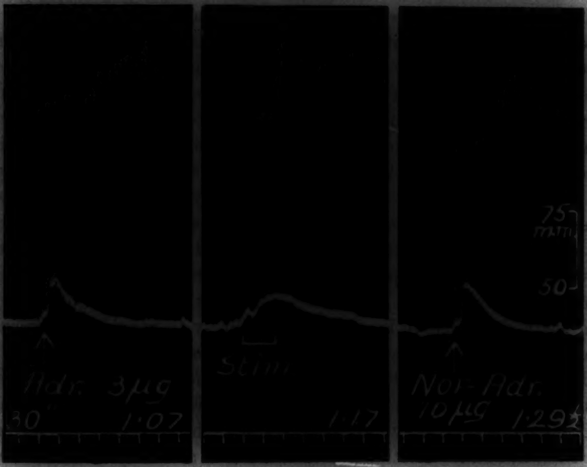

Fig. 8. Decerebrate cat. Vagi cut. Duodenum and blood pressure. Stimulation of hepatic nerves for 1 min. Adrenaline and noradrenaline, intravenous.

noradrenaline rather than adrenaline, but the difference between the two substances was not great and the evidence was not conclusive. Fig. 8 shows an exceptional experiment in which liver sympathin, adrenaline, and noradrenaline all caused an increase in the tone of the intestine. At an earlier stage this intestine had been responding to all three stimuli with inhibition followed by a small contraction.

DISCUSSION

We have never failed to confirm the experimental results obtained by Cannon and his co-workers. Some of these results can, however, be interpreted in more than one way. The effects on the blood pressure depend on a number of factors and can be interpreted in terms of local effects in the liver, associated with the liberation of adrenaline. Some of the discrepancies between quick injections of adrenaline and the effects of stimulating hepatic nerves can be matched by similar discrepancies between quick and slow injections of adrenaline, but we have not been very successful in an attempt to reproduce all the effects of liver sympathin with slow injections of adrenaline. This may be because of the difficulty of choosing the appropriate dose and rate of injection, but the results are more easily explained on the theory that liver sympathin is not adrenaline. Its properties are not those postulated for pure sympathin E since it sometimes has inhibitory effects on the intestine and the virgin uterus. If the stimulation of adrenergic nerves liberates two substances with purely excitor and inhibitor effects, there is no convincing evidence that either of them is ever liberated without the other. Whenever we have made the comparison, the effects of liver sympathin have resembled those of noradrenaline more closely than those of adrenaline, and there is no evidence against the theory that liver sympathin is noradrenaline. Recent work on transmethylation makes the natural occurrence of noradrenaline more probable than it was.

According to Barger & Dale (1910) inhibitor actions are feeble in amines with no *N*-methyl group. There are presumably various such amines with properties like those of liver sympathin. We have some evidence that tyramine has the appropriate properties. The results could be explained in the theory that stimulation of the hepatic nerves caused the liberation of noradrenaline at the rate of about 10 μg./min. or tyramine at the rate of about 1 mg./min.

The methods which we have used are unlikely to provide convincing evidence of the chemical structure of liver sympathin. The experiments of Euler (1946*a*, *b*) represent a new approach to this question. He has found that extracts of various organs contain a substance with properties like those of noradrenaline or dihydroxynorephedrine. This substance may well prove to be sympathin E, and its isolation and identification are likely to throw new light on the problem.

SUMMARY

1. A critical study has been made of the theory that the substance liberated by the hepatic nerves in a cat (liver sympathin) is not adrenaline.

2. Some of the evidence for this theory is unconvincing, but it provides the simplest explanation of the results as a whole.

3. The fact that liver sympathin may inhibit the virgin uterus and intestine has been confirmed. There is no good evidence that either of the hypothetical substances, sympathins E and I, is ever liberated without the other.

4. The results are compatible with the theory that liver sympathin is noradrenaline or perhaps tyramine, but it is probably impossible to identify it convincingly by the methods which have been used.

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THE ACTION OF CHEMICAL VESICANTS ON CHOLINESTERASE

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Towards the end of 1941 information began to accumulate suggesting that some of the systemic effects produced by certain chemical warfare vesicants had a resemblance to the muscarine-like actions of acetylcholine. In view of this, and on account of the physiological importance of acetylcholine, both in synaptic and neuro-muscular transmission as well as in its parasympathomimetic effects, it was decided to investigate the action of certain vesicant substances on cholinesterase.

The work described here has already been submitted to the Ministry of Supply in three reports (Thompson, 1942*a*, *b*, 1943).

At the outset of the work it was decided to investigate a 'tissue' cholinesterase rather than the esterase present in the serum, as it was thought that considerable differences in properties might exist between the enzyme present locally in the neighbourhood of nerve endings, where rapid inactivation of acetylcholine is essential, and the enzyme of the serum. Alles & Hawes (1940) and Glick (1941) had already reported on differences in the specificity and other properties of cholinesterase preparations from different sources, and the view that cholinesterase activity is exhibited by at least two types of enzyme (a specific cholinesterase and a non-specific pseudo-cholinesterase) has since been amply confirmed (Richter & Croft, 1942; Mendel & Mundell, 1943; Mendel, Mundell & Rudney, 1943; Mendel & Rudney, 1943).

For this reason the pigeon brain was used as the source of the enzyme in the initial experiments, rat skin and guinea-pig serum being used later. In this connexion it is of interest to note that Nachmansohn & Rothenberg (1944) have claimed that nervous tissue, unlike other tissues, contains only the specific cholinesterase.

METHODS

Vesicants

The following vesicants, kindly supplied by the Chief Superintendent, Experimental Station, Porton, have been studied:

$\beta\beta'$-Dichlorodiethyl-*N*-methylamine.
Mustard gas ($\beta\beta'$-dichlorodiethylsulphide).
Trichlorotriethylamine.
Carbomethoxy-β-chloroethylnitrosamine.
Lewisite (β-chlorovinyldichlorarsine).
Phenyldichlorarsine.
Ethyldichlorarsine.

Enzyme preparations

(1) *Brain.* The enzyme was prepared from pigeon brain by a modification of the method of Nachmansohn (1939) by grinding with bicarbonate-Ringer's solution (containing calcium), one brain being finally dispersed in 30 ml. Ringer's solution. The dispersion was then filtered through muslin at room temperature, and 0·3 ml. of the filtrate used for each test.

(2) *Skin.* Rat skin, clipped free from fur, was removed from the animal immediately after killing by decapitation. The underlying fat and connective tissue were dissected away with a scalpel, and the skin sliced with a razor. 100–150 mg. of slices were used for each test.

(3) *Serum.* Blood was drawn by cardiac puncture from guinea-pigs, and the serum, separated by centrifuging, was diluted with bicarbonate buffer (pH 7·4) so that 1·0 ml. of the dilution contained 0·15 ml. serum. Activity was determined on 1·0 ml. aliquots of the dilutions.

Estimation of esterase activity

Esterase activity was determined by Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis is measured by CO_2 evolution from bicarbonate buffer.

All measurements were carried out at pH 7·4 and 38° C., the gas space in the flasks being filled with 95% N_2 + 5% CO_2 immediately before placing the flasks in the bath.

In all cases, except where stated, 8 mg. of acetylcholine chloride (British Drug Houses) were tipped in from the side-bulbs after temperature equilibration to give a final concentration of 0·015 M. In most cases the vesicant, dissolved in bicarbonate buffer, was added to the main flask immediately after the addition of the enzyme, i.e. about 15–20 min. before the acetylcholine was tipped in.

In each experiment flasks were set up to control the rate of non-enzymic hydrolysis of acetylcholine, and in the earlier experiments to control also the rate of CO_2 evolution by the preparation in the absence of added acetylcholine. Both non-enzymic hydrolysis and acid production by the enzyme preparation alone were in all cases small.

The manometers were in most cases read at 10 min. intervals, measurements in all experiments being carried out in duplicate.

RESULTS

Effects on brain cholinesterase

The initial experiments of this series were carried out with ββ'-dichlorodiethyl-*N*-methylamine. Both the free base and the hydrochloride were studied, the compound in each case being dissolved in bicarbonate buffer immediately before addition to the enzyme. It will be seen (Fig. 1) that a concentration of approximately 0·15 mM. of the hydrochloride (or slightly more of the free base) produces approximately 50% inhibition of enzymic activity.

In the experiments summarized in Fig. 1 the enzyme was in contact with the poison for 5 min. at room temperature (during which time the flasks were filled with gas), followed by 10 min. in the bath (for temperature equilibration) before the acetylcholine was tipped in. If, however, acetylcholine was also present from the time of commencement of poisoning the degree of inhibition was markedly reduced; in the experiments summarized in Table 1 acetylcholine and the inhibitor were in the main compartment of the flasks, the enzyme being tipped in from the side-bulbs after temperature equilibration.

By contrast with the results shown in Fig. 1, in which 0·17 mM. ββ'-dichlorodiethyl-*N*-methylamine produced over 50% inhibition, it will be seen that this concentration produced only 10% inhibition if 3 mg. acetylcholine were present when the poison was tipped in, while 30 mg. acetylcholine protected against concentrations as high as 1·34 mM. $\beta\beta'$-dichlorodiethyl-*N*-methylamine.

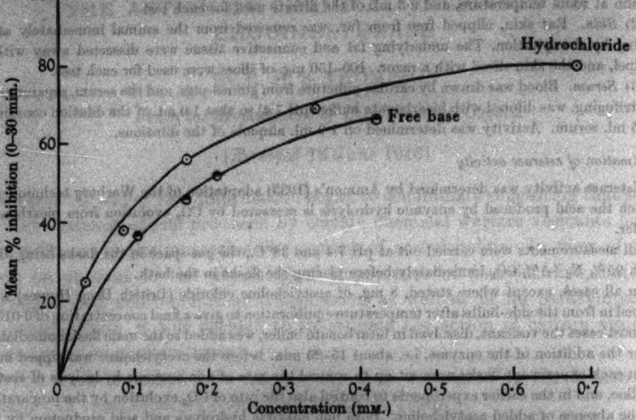

Fig. 1. Inhibition of brain cholinesterase by $\beta\beta'$-dichlorodiethyl-*N*-methylamine.

It has been shown by Matthes (1930) that the inhibitory effect of eserine on cholinesterase can be completely reversed by dialysis. Since, with the preparations used here, the enzyme is largely present in the insoluble, centrifugable fraction of the brain dispersion, it was decided to determine whether the enzyme, after poisoning by the vesicant, could be reactivated by centrifugation and resuspension of the precipitate in fresh bicarbonate buffer. A brain preparation was therefore divided into two parts, one of which was centrifuged for 5 min., and the precipitate resuspended in the same volume of bicarbonate buffer. The percentage inhibitions produced by 0·17 mM. $\beta\beta'$-dichlorodiethyl-*N*-methylamine on the fresh and centrifuged fractions were determined and compared with the inhibition remaining in a fresh preparation poisoned with the same amount of the vesicant, but centrifuged after 15 min. and resuspended

TABLE 1. Effect of concentration of acetylcholine on the toxicity of $\beta\beta'$-dichlorodiethyl-*N*-methylamine to brain cholinesterase, the substrate being present from the commencement of poisoning

Concentration of inhibitor	Mean % inhibition (0–30 min.) at the initial concentrations of substrate stated (mg. acetylcholine chloride/3 ml.)		
(mM.)	3	15	30
0·17	10	—	—
0·68	24	—	—
1·34	34	4	1

in fresh bicarbonate buffer. Table 2 shows the result of this experiment, which suggests that under these conditions some degree of reversal of poisoning can be brought about by resuspending the poisoned enzyme in a fresh medium.

TABLE 2. Percentage inhibition produced by 0·17 mM. ββ′-dichlorodiethyl-*N*-methylamine, (*a*) on a fresh preparation of brain cholinesterase; (*b*) on preparation (*a*) after centrifuging and resuspending in its original volume of $NaHCO_3$ buffer; (*c*) on preparation (*a*), centrifuged and resuspended in the same volume of fresh buffer after 15 min. poisoning

	Percentage inhibition			
	0–10 min.	10–20 min.	20–30 min.	Mean
(*a*) Fresh enzyme	59	53	59	57
(*b*) Centrifuged enzyme	66	66	71	68
(*c*) Fresh enzyme, poisoned and then centrifuged and resuspended	19	24	26	23

It has also been shown that the non-vesicant ββ′-dihydroxydiethyl-*N*-methylamine is non-toxic to brain cholinesterase, a concentration of 0·56 mM. producing only 3% inhibition.

Goodman & Gilman (1942), who remarked on the close similarity between the effects of ββ′-dichlorodiethyl-*N*-methylamine and the muscarine-like actions of acetylcholine, have also stated that trichlorotriethylamine frequently produces salivation, vomiting and defaecation in poisoned animals. Trichlorotriethylamine and two other non-arsenical vesicants, mustard gas and carbomethoxy-β-chloroethylnitrosamine were therefore also tested with respect to their action on brain cholinesterase.

Table 3 shows the percentage inhibitions produced by varying amounts of these compounds, together with results obtained with ββ′-dichlorodiethyl-*N*-methylamine for comparison.

TABLE 3. Inhibition of brain cholinesterase produced by four non-arsenical vesicants. (Numbers in brackets denote number of experiments)

Vesicant	Mean % inhibition produced by the concentration (in μg./3 ml.) stated			
	50	100	200	500
ββ′-Dichlorodiethyl-*N*-methylamine HCl	43 (3)	56 (6)	69	—
Trichlorotriethylamine HCl	22	55 (4)	75	—
Carbomethoxy-β-chloroethylnitrosamine	—	12	20	36 (4)
Mustard gas	—	—	—	28 (7)

From Table 3 it will be seen that trichlorotriethylamine is also an active inhibitor of this enzyme, a concentration of 0·15 mM. producing rather more than 50% inhibition. Carbomethoxy-β-chloroethylnitrosamine and mustard gas are less potent inhibitors, concentrations of approximately 1 mM. being required to produce inhibitions of 36 and 28% respectively.

It was of interest to determine next whether the arsenical vesicants also inhibited cholinesterase. Before commencing the work described above on ββ′-dichlorodiethyl-*N*-methylamine, it had been shown in this laboratory that sodium arsenite is highly toxic to brain cholinesterase. As early as 1939 Massart & Dufait had stated that horse-serum cholinesterase is inhibited by arsenite, and with a tissue cholinesterase (brain) I have found that concentrations of arsenite as low as 0·17 mM. produce approximately 50% inhibition.

The results obtained with sodium arsenite and the arsenoxides derived from phenyldichlorarsine, ethyldichlorarsine and lewisite are given in Table 4.

TABLE 4. Inhibition of brain cholinesterase by arsenicals.
(Numbers in brackets denote number of experiments)

Arsenical	Mean % inhibition produced by the concentration (in μg./3 ml.) stated			
	50	100	200	500
Sodium arsenite	52	69 (4)	—	—
Phenyldichlorarsine	19	30	40 (2)	57 (3)
Ethyldichlorarsine	—	—	—	35 (2)
Lewisite	—	2	2	19 (5)

The three organic arsenicals are each less toxic to this enzyme than sodium arsenite, lewisite in particular being a poor inhibitor at the concentrations studied; in each case, however, a significant inhibition is produced by concentrations of approximately 1 mM. (500 μg./3 ml.). Using still higher concentrations of lewisite (M./250) Mackworth (1942) obtained 30% inhibition of the serum enzyme.

Cholinesterase activity of normal and contaminated skin

The presence of a specific cholinesterase in skin has already been described (Thompson & Whittaker, 1944), and the effect on this enzyme of contamination of the skin with vesicants was investigated as follows:

Young, white rats, weighing from 30 to 70 g., were used. Two areas of skin, each approximately 10–15 sq. cm. on each side of the animal, were closely clipped free from fur with scissors, the animal being kept lightly anaesthetized with ether. After the effects of the anaesthetic had passed off, one of the clipped areas was contaminated with the vesicant, which was dropped on to the skin from a pipette and spread over the area with a glass rod. The rat was then returned to its cage and kept under observation for 1 hr., at the end of which time it was killed by decapitation. Both areas of clipped skin, contaminated and uncontaminated, were then removed, and their cholinesterase activities compared. By this means the enzymic activity of the contaminated skin was always compared with that of normal skin from the same animal, thus obviating any differences in cholinesterase content that might exist between the skins of different animals.

Three non-arsenical vesicants, mustard gas, ββ′-dichlorodiethyl-*N*-methylamine and carbomethoxy-β-chloroethylnitrosamine, were investigated along these lines. In each case, except the first two experiments with mustard gas, contamination was carried out with the undiluted vesicant. A series of determinations of the percentage inhibition of cholinesterase activity in the skin of different animals contaminated with these vesicants is given in Table 5. It will be seen that contamination of the skin with carbomethoxy-β-chloroethylnitrosamine produces an inhibition of enzymic activity in the affected skin amounting to 79–92%. Contamination with mustard gas or ββ′-dichlorodiethyl-*N*-methylamine yields more variable results, in the case of mustard gas inhibitions up to 50% (mean value = 33%) being obtained, and in the case of ββ′-dichlorodiethyl-*N*-methylamine up to 40% (mean value = 29%).

Table 5. Percentage inhibition of cholinesterase activity in rat skin contaminated with non-arsenical vesicants

(Contamination = 1 hr. with undiluted vesicant except where otherwise stated.)

Carbomethoxy-β-chloroethylnitrosamine	Mustard gas	ββ′-Dichlorodiethyl-*N*-methylamine
80	22*	39
84	37*	12
79	50	39
92	21	27
	27	28
	47	
	30	
Mean = 84	33	29

* 2 hr. contamination with 50% mustard gas in ethanol; control skin painted with ethanol only.

In all cases marked erythema of the contaminated skin was observed at the time of killing, and on removing the skin considerable oedema, often partly gelatinous in nature, was present in the subcutaneous tissues. The rats contaminated with mustard gas or ββ′-dichlorodiethyl-*N*-methylamine invariably showed profuse salivation and lachrymation for some time before killing. In three of the experiments the tears were seen to be stained red; a small quantity of the tears was therefore collected and examined in ultra-violet light, when they were seen to show a pronounced red fluorescence; the possible significance of this finding is discussed later. In addition to these signs the rats contaminated with ββ′-dichlorodiethyl-*N*-methylamine were considerably collapsed at the time of killing.

In the experiments with carbomethoxy-β-chloroethylnitrosamine, on the other hand, excessive salivation and lachrymation were not present, and the rats were in good condition at the end of the hour; locally, however, erythema and considerable oedema were invariably present.

Owing to the rapid onset of collapse and death after contamination of a large area of skin with one of the arsenical vesicants, it was not possible by this method to investigate the effects of similar contaminations with any of these compounds. One experiment, however, was carried out with sodium arsenite in order to determine whether the cholinesterase in skin is poisoned in vitro by a trivalent arsenical; this was found to be the case, the addition of 0·17 mM. sodium arsenite to normal rat skin slices resulting in 36% inhibition of activity.

In vivo effects on level of serum cholinesterase

In view of the rapid onset of signs of systemic intoxication in animals heavily contaminated with mustard gas, a comparison was made of the serum cholinesterase level in normal animals and in animals after heavy contamination of the skin with this vesicant.

Owing to the relatively low cholinesterase content of rat blood (Stedman, Stedman & White, 1933), guinea-pigs have been used throughout.

Blood was drawn by cardiac puncture from the living animal, and esterase activity determined on a dilution of the serum obtained as already described.

In each experiment the activity of normal serum, freshly obtained, was compared with that of serum from the contaminated animal, a total of twelve normal and twelve experimental animals being examined. The experimental animals were contaminated on the clipped skin of the back with 100 mg. mustard gas/kg. body wt., the serum being drawn 18–50 hr. after contamination, i.e. at a time when all the animals were showing signs of systemic poisoning (excessive salivation).

The results, summarized in Table 6, show that there is a significant fall in the cholinesterase level of the serum of guinea-pigs following contamination with mustard gas. No evidence was obtained suggesting that the fall was any greater after 50 hr. than after 18 hr.

TABLE 6. Serum cholinesterase level in normal guinea-pigs and in guinea-pigs contaminated with mustard gas

(Contamination = 100 mg. mustard gas/kg. body wt. Serum tested 18–50 hr. later.)

Enzymic activity ($\mu l.\ CO_2$/ml. serum/min.)	
Normal	Contaminated
28·2	16·5
31·2	20·2
26·5	10·3
39·6	17·8
21·6	19·7
23·1	17·7
16·1	14·8
23·2	15·5
24·1	15·8
29·7	13·9
31·0	13·4
31·0	13·7
Mean = 27·1 ± 1·75	15·8 ± 0·81

Difference of means = 11·3 ± 1·93

$t = 5{\cdot}85$ ($n = 22$); $P = 0{\cdot}001$.

Evidence of muscarine-like effects produced by vesicants

Chromodacryorrhoea

Before considering the wider experimental evidence pointing to the development of muscarine-like effects in animals poisoned with various vesicants, the significance of the red tears secreted by contaminated rats, mentioned earlier, will be discussed.

It has been repeatedly noticed in this laboratory that white rats poisoned with mustard gas, given either by injection or by application to the skin, frequently develop in the course of the next 4 or 5 days a characteristic condition of the eyes, consisting of an accumulation of dried reddish brown matter around the palpebral margins. In the course of the experiments already described in this paper, it was noticed that three rats that had received heavy contamination of the skin, one with mustard gas and two with $\beta\beta'$-dichlorodiethyl-*N*-methylamine, were freely secreting deep red tears 1 hr. after contamination; as already mentioned these tears showed a pronounced red fluorescence in ultra-violet light.

It seemed therefore as if these contaminated animals were showing the condition of chromodacryorrhoea recently described by Tashiro, Smith, Badger & Kezur (1940). Freud (1933), quoted by Selye (1937), observed that injected acetylcholine causes white rats to shed tears which he described as being tinged with blood. Tashiro, Smith, Badger & Kezur (1940) have extended these observations, and have described a flow of red tears commencing 2–5 min. after intraperitoneal injection of acetylcholine, usually following salivation and the secretion of clear tears.

In a more recent paper Tashiro, Badger & Younker (1940) have shown that Freud's assumption that the red colour of the tears is due to haemoglobin is incorrect. The pigment possesses absorption bands close to but not identical with those of oxyhaemoglobin; moreover, the lachrymal pigment shows a strong red fluorescence. They have suggested that the pigment may be identical with, or related to, the porphyrin in the Harderian glands of rats described by Derrien & Turchini (1924).

It was decided therefore to compare the ocular condition developing after contamination of rats with mustard gas or $\beta\beta'$-dichlorodiethyl-*N*-methylamine with the chromodacryorrhoea produced by acetylcholine.

In confirmation of the earlier work it was found that red tears may be regularly and rapidly produced in white rats by the injection of acetylcholine. Acetylcholine chloride, 5–6 mg./kg. body wt. injected subcutaneously, produced a copious flow of dark red, opaque tears commencing 3–4 min. after the injection; the flow was maintained for a period of 5–15 min.

Two rats were next given 5 mg. eserine sulphate/kg. body wt. subcutaneously; profuse lachrymation and salivation, accompanied by twitching movements, developed and were followed, half an hour after the injection, by a copious excretion of the typical red opaque tears.

Two rats received, by subcutaneous injection, 47 and 55 mg. mustard gas (dissolved in cellosolve)/kg. body wt. respectively. On the following day the rat that received the larger dose began to secrete red tears, and died late that afternoon. On the second morning after the injection the other rat was found to be secreting red tears profusely; a large amount of dried dark red matter was attached to the lid margins, while the inner canthus of each eye was filled with red fluid. The secretion of red tears was continued throughout the 2nd and 3rd days, and on the morning of the 4th day the animal was found dead.

50 mg. ββ′-dichlorodiethyl-*N*-methylamine were next injected into two rats. A flow of red tears occurred in both animals, commencing about 50 min. after the injection and about 10–15 min. before death.

Both mustard gas and ββ′-dichlorodiethyl-*N*-methylamine can therefore give rise to a secretion of red tears in white rats, comparable to that produced by injected acetylcholine or eserine.

Other evidence

As pointed out earlier, considerable experimental evidence exists in both English and American literature pointing to the slow development of muscarine-like effects in animals after contamination with various vesicants.

The onset of lachrymation, salivation and diarrhoea after application of ββ′-dichlorodiethyl-*N*-methylamine has been described by Cameron & Short (1942*a*), while in America Goodman & Gilman (1942) have described the fall in blood pressure, salivation, meiosis and increased gastro-intestinal activity that occurs after poisoning with this compound.

The case for the development of muscarine-like effects after administration of mustard gas does not seem to have been stressed. Apart from the haemoconcentration, however (Cameron, 1941), Cameron & Short (1942*b*), in confirmation and extension of work by Florey (1940), have described the acute and often haemorrhagic gastritis, associated with haemorrhages in the small and large intestine after application of mustard gas to the skin. Courtice & Cameron (1942) investigated the blood changes, and concluded that 'a picture of so-called shock is produced as a result of the loss of plasma, probably mainly local at the site of application of the mustard gas, and secondly another train of functional changes develops and continues to progress long after the initial shock has subsided. The fundamental lesion in this latter disturbance appears to be a gastro-enteritis which causes diarrhoea and an increased salivation.'

DISCUSSION

The ability to inhibit brain cholinesterase has been shown to be, in varying degrees, a common property of all the vesicants studied here. Three of them, when applied to the skin of living animals, have also been found to reduce the cholinesterase activity of this tissue.

In this respect the enzyme in horse serum is in striking contrast to that in brain; using the serum enzyme Mackworth (1942) obtained only 5% inhibition of activity with 0·33 mM. ββ′-dichlorodiethyl-*N*-methylamine. In vivo, however, in animals contaminated with lethal amounts of mustard gas, a lowering of the level of serum cholinesterase has been found, although no attempt has been made to discover if this is a direct effect of the absorbed mustard gas or products of mustard gas, or whether it is secondary to other changes brought about by the vesicant.

The muscarine-like actions of certain of the nitrogenous vesicants have already been commented on, and evidence has also been brought forward showing that, like acetylcholine or eserine, mustard gas and $\beta\beta'$-dichlorodiethyl-*N*-methylamine can bring about the secretion of red tears when injected into white rats.

In view of these findings it is suggested that some of the systemic effects produced by certain of the vesicants may be due, in part at any rate, to an inhibition of the cholinesterase at cholinergic nerve-endings. A failure to hydrolyse liberated acetylcholine could well account for certain of the changes observed. In this connexion it is also of interest to compare the leucopenia observed by Cameron (1941) in animals poisoned with mustard gas with that observed by Dale & Laidlaw (1919) after the slow infusion of acetylcholine; in both cases the polymorphonuclear cells were more affected than the lymphocytes.

It is clear from what is now known about the effects of these vesicants that this is only one of several biochemical actions exerted by them at these concentrations. Moreover, by comparison with eserine they are relatively weak inhibitors of cholinesterase in vitro. It is not suggested therefore that any effect that they may exert on the cholinesterase in skin is playing a central role in the pathogenesis of vesication, but since all the vesicants studied produce systemic effects which could in part be explained by cholinergic activation it is of interest, despite the wide range of effects produced by them on cholinesterase in vitro, to review the known facts concerning the local effects of an accumulation of acetylcholine in the skin, such as might result from inhibition of the cholinesterase.

In a study of urticaria of nervous origin Grant, Bruce Pearson & Comeau (1936) concluded that the condition was brought about by the release of acetylcholine in the skin as a result of stimulation of cholinergic nerve fibres. This conclusion was based partly on the results observed after the electrophoretic introduction into the skin of various choline derivatives and pharmacologically related substances. It was found that the introduction of carbaminoylcholine into the skin of these patients regularly produced an urticarial attack. Acetylcholine was less certain in its action unless eserine was also present; whealing, which could be abolished by atropine, also occurred in response to warming the legs.

According to these authors acetylcholine with eserine, or carbaminoylcholine, introduced electrophoretically into the skin of normal subjects produces only a local reddening, lasting 10–15 min., without flare or wheal.

Alexander, Elliott & Kirchner (1940), on the other hand, claim that eserine is a powerful urticariogenic substance, although few experimental details are given. In view of these divergent results a few rough experiments were carried out in this department with acetylcholine, carbaminoylcholine and eserine. Using a 2 mA. current from a 45 V. battery for either 2 or 3 min. it was found that 1:100 carbaminoylcholine or eserine sulphate produces whealing of the skin; the response was more marked with eserine, wheals commencing to appear 2 min. after removing the electrode, rapidly becoming confluent over the entire area covered by the electrode, and persisting for 1½–2 hr. Eserine (1:1000) was also found to produce wheals; no higher dilution was tested. Acetylcholine (1:100) caused intense erythema, lasting for about 1 hr., and pilo-erection, but no wheals. Although these lesions are obviously fundamentally different from the necrosing blisters produced by chemical vesicants, it is regarded as significant that a recognized inhibitor of cholinesterase (eserine) can cause erythema and whealing of skin when introduced into it.

SUMMARY

1. It has been shown that a number of chemical vesicants, both arsenical and non-arsenical, are inhibitors of brain cholinesterase.

2. When applied to the skin of rats, mustard gas and two other nitrogen-containing vesicants have also been shown to produce an inhibition of the cholinesterase activity of the contaminated skin.

3. There is a significant reduction in the cholinesterase content of the serum of guinea-pigs after heavy contamination with mustard gas.

4. Both mustard gas and $\beta\beta'$-dichlorodiethyl-*N*-methylamine, when injected into white rats, can give rise to the secretion of red tears, comparable to those produced by injected acetylcholine or eserine.

5. The possible significance of these findings in connexion with the systemic and local effects produced by contamination of the skin with chemical vesicants is discussed.

I should like to express my gratitude to Prof. R. A. Peters, M.C., F.R.S., for his advice and interest throughout this work. My thanks are also due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, and to Miss M. R. Kempson for technical assistance.

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VITAMIN A AND BONE GROWTH: THE REVERSIBILITY OF VITAMIN A-DEFICIENCY CHANGES

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In a series of publications (Mellanby, 1926, 1931, 1933, 1934, 1935) it has been shown that a deficiency of vitamin A in the diet of growing animals causes widespread nerve degeneration, both peripheral and central. Of the cranial nerves, the olfactory, the optic, the trigeminal (first and second divisions) and the auditory (both cochlear and vestibular divisions) are specially liable to suffer destructive changes. For the most part, under the conditions of these experiments, the cranial motor nerves escape. In the spinal cord, afferent nerves having their origin in the posterior root ganglia are often destroyed, together with their neurones passing into the cord. In addition, some of the endogenous ascending fibres are affected, especially the anterior and posterior cerebellar tracts.

A cause of this widespread degeneration was suggested when the examination of serial sections of the labyrinth and its capsule revealed that vitamin A deficiency results in a large bone hypertrophy which injures or destroys the nerve fibres and the cells of the spiral ganglion by pressing on the VIII nerve (Mellanby, 1938). Other observations made it clear that in vitamin A-deficient animals the whole of the nervous system was under greatly increased pressure (Mellanby, 1939*a*); the brain and spinal cord were closely packed into the cranium and spinal canal, and it soon became evident that, because of their abnormal growth, the skull and vertebrae had lost their primary function of protecting the nervous system and had become destructive agents.

Later (Mellanby, 1939*b*, 1941, 1943) descriptions were given of the abnormal bone growth near the spinal and cranial nerve cells, and in the Croonian Lecture of the Royal Society in 1943 a more detailed analysis of the abnormal growth in two positions, namely, the basi-occipital bone and the vertebral column, was presented (Mellanby, 1944). It was shown that the bone dysplasia which resulted from vitamin A deficiency and had such grave effects on the nervous system could be largely, if not entirely, explained by altered osteoclastic and osteoblastic activity.

A question raised in the Croonian Lecture was—how would the osteoclasts and osteoblasts of vitamin A-deficient puppies react if the animals were later given the vitamin? Would the cell activity be again reversed, and, if so, would the normal shape of the bones be restored? In a paper read before the Physiological Society in December 1945, this was answered, in part at least. It was shown that, under the conditions tested, the bone changes resulting from vitamin A deficiency are reversible, and that soon after the addition of the vitamin the osteoclasts and osteoblasts again become active in their normal positions, the osteoclasts especially being very numerous and active, as if making a special effort to remove the superfluous bone laid down or not absorbed in the period of vitamin A deficiency. The bones tend to return to normal, though no definite decision can yet be given as to whether they ever do so completely. Whether the small bone abnormalities seen in adult A-deficient dogs can be corrected by the addition of vitamin A to the diet is not yet known.

The dysplasia due to vitamin A deficiency appears to involve all the bones of the body to a greater or less extent. In previous reports attention has been concentrated mainly on the bones near the nervous system because it is their abnormal growth which causes such devastating effects on the animal's life. In the present publication also the study is confined to bones of the skull and, even so, only a few examples are given to illustrate the different ways in which growth of bone takes place and how these various processes are affected by the absence and restoration of vitamin A. It may be added, however, that sufficient study has been made of the action of vitamin A in the growth of other bones, including the long bones and pelvic bones, to ascertain that the vitamin plays a controlling part in what might be called the moulding of all bones.

An investigation of the present kind cannot be made without revealing some of the complexities of normal bone growth. The chief point of interest in this publication is in the study of the reparative or recovery stage of bone growth, especially of the cranial bones, when vitamin A is restored to an animal after a period of deficiency. The three bones discussed are (*a*) the basi-occipital, (*b*) the labyrinthine capsule and (*c*) the sphenoid bone. They were chosen because they represent three different types of bone growth, and it was thought desirable to describe what happens in each when vitamin A is largely removed from the diet and so ultimately from the animal's body and, secondly, when vitamin A is restored. It was hoped that by studying bones with different responses to the presence and absence of vitamin A it might be possible to get some insight into its mode of controlling bone growth. All that can be said at present is that vitamin A, when given in proper amounts under the experimental conditions adopted, ensures that bone growth or bone moulding proceeds along correct, predestined and safe lines and that, when the vitamin is deficient, growth continues in an orderly but wrong way, which leads to injury or even destruction of the nervous tissue and ultimately to the death of the animal. The restoration of vitamin A to the diet of the deficient animal quickly brings about a response in the bone cells directed to correct the dysplasia resulting from the absence of the vitamin.

EXPERIMENTAL METHODS

A full account has been previously given (Mellanby, 1931) of the symptoms which develop in puppies on diets similar to the one used in the experiments now to be described, which is as follows: wheatmeal bread 30–280 g., salt 1–2 g., baker's yeast 5% of cereal, separated milk powder 20–25 g., lean meat 15 g., peanut oil 10 c.c., ascorbic acid 5 mg., vitamin D_2 200–2000 i.u.

The wheatmeal flour from which the bread was made varied between 80 and 85% extraction at different times during the period of the investigation and contained a trace of carotene. Similarly, the meat part of the ration no doubt included a small quantity of vitamin A. The diet was therefore not totally devoid of the vitamin or its precursor.

For the sake of brevity the term +A dog is used to denote one having an abundant supply of vitamin A, and −A dog for one receiving only the basal diet, and recovery dog or −A+A dog for one having at first the basal diet only, and later the same diet supplemented by abundant vitamin A.

The bones of −A dogs become thickened and cancellous as compared with those of +A litter-mates. To produce compact bones more calcium is needed. For instance, if extra calcium as calcium carbonate (1 g. daily) is added to the above basal diet, the bones of both +A and −A animals are less cancellous than without such addition, but the −A bone is still much thicker than the +A. Calcium is not, therefore, the controlling factor as regards bone hypertrophy (Mellanby, 1944).

In the case of +A diets, it has been usual to add to the basal diet vitamin A (2000–5000 i.u. daily) in the form of a proprietary preparation of mammalian liver oil or as pure vitamin A acetate. This applies both to the control +A dogs and to the recovery animals.

The histological side of this investigation, involving serial sections of large blocks of decalcified bone cut at carefully chosen angles in relation to the nervous tissue, was long and tedious. The blocks were usually embedded in celloidin, one in every ten sections being stained and mounted. One of the difficulties in this work was that bone overgrowth, especially in complicated structures, often made histological examination of identical positions in +A and −A dogs impossible. The difficulty was partly overcome by examining serial sections; for instance, Pl. 6, Fig. 1*a* (+A) shows the internal auditory meatus and the helix of the cochlea, but although Pl. 6, Fig. 1*b* (−A) shows a similar portion of the helix, it is necessary to examine other sections to trace the tortuous course of the internal auditory meatus of this animal.

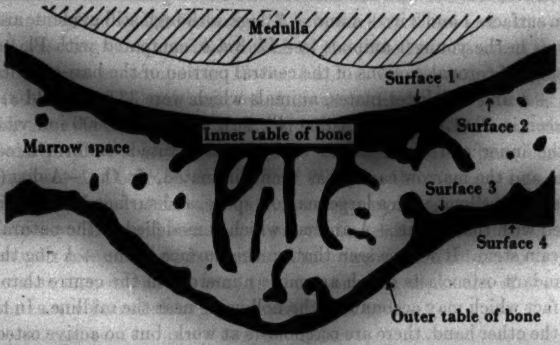
Most of the results described in this paper refer to three litter-mate puppies, the experimental feeding of which started at the age of 7 weeks and continued in the case of the +A and −A dogs for 26 weeks. In the case of the third dog (referred to as the recovery or −A+A dog) the feeding of the −A diet lasted for 24 weeks and of the +A recovery diet lasted for 23 days, making the experimental feeding period 27 weeks in all.

Less complete examinations were made on another three animals. The feeding period of the +A and −A dogs lasted from the age of 6 to 20 weeks, i.e. for 14 weeks, and the third (recovery) animal then had vitamin A added to the diet for 13 days. At the end of the experiment these animals were only 20–22 weeks old, as compared with the first set of three, which were about 33 weeks old at death.

For special points, bone tissues were examined from a number of other experimentally fed dogs, usually in order to see what happened to the bones of dogs of different ages, and in such cases time was saved by embedding decalcified bones in paraffin wax instead of celloidin. Serial sections were not prepared.

Although bone changes in both the recovery animals (−A+A) give similar pictures as regards the reaction of osteoclasts and osteoblasts, the age of the animals greatly affects the cell activity observed in +A and −A dogs. It is probably not remarkable that this should be so, but the rapidity with which the points of maximum growth vary in bones at different ages was rather perplexing and added greatly both to the difficulty of understanding and of describing the detailed incidents of bone growth.

Reference will often be made to the osteoclasts and osteoblasts. In the case of the osteoclasts it is usually easy to count them in sections of bone when they are active and to judge of their relative activity by their size and shape and by the bone lacunae they form when engaged in removing bone. Early in the work it seemed difficult to understand how bone could have osteoblasts and osteoclasts respectively on opposite surfaces and at a later stage could have these same surfaces covered by the other type of cell. It is now evident that there are either active or inactive bone cells of both types present on most bone surfaces in the period of rapid growth, and that different circumstances, for example, the presence or absence of vitamin A, make a different type of cell active. Flattened inactive (Pl. 1*a*) and enlarged active osteoblasts (Pl. 1*b*) can be seen on many bone particles. Inactive flattened osteoclasts also can be seen on surfaces where, under other circumstances, they are apparently roused to activity and become rounder and more clearly multinuclear in the process. Osteoclasts of both forms can be seen in Pl. 1*c–f*. Occasionally active osteoclasts appear in great numbers on some bone surfaces where there was previously no evidence of inactive osteoclasts. In such a case it might be that these active osteoclasts were either transferred to the surface or possibly formed there from other precursors, i.e. possibly by a fusion of osteoblasts. This latter suggestion is not based on any definite observation.



Text-fig. 1. Diagram of general structure of basi-occipital bone. Surfaces have been numbered to simplify description in text. Surface 1 is the bone surface adjacent to the brain stem; it is separated from the nervous tissue by the closely attached periosteum (dura mater) and other membranes. Surface 2 is the marrow surface of the same inner table of bone. Surface 3 is the marrow surface of the outer table of bone. Surface 4 is the outer surface of the outer table of bone.

RESULTS

Basi-occipital bone

To simplify the description of this bone, reference will be made where possible to surfaces 1–4. Surfaces 1 and 2 limit the inner table of bone, 3 and 4 the outer table. Surface 1 is adjacent to the central nervous system, 2 and 3 are adjacent to marrow, and 4 is the external surface of the bone (see Text-fig. 1).

In the Croonian Lecture of 1943 it was shown that a −A diet produced a thick cancellous basi-occipital bone, the abnormal thickness of which was caused largely by lack of absorption of surface 1; indeed, actual deposition of additional bone on this surface (Text-fig. 1) could be seen at certain early stages of growth on these deficient diets. It appeared that absorption was greatly reduced or had even ceased because the osteoclasts, normally active and abundant in this position during growth, gave way to active osteoblasts. Conversely, on surface 2 osteoblastic activity had ceased and been succeeded by osteoclastic activity. This is shown in Pl. 2*a*, *b*, photomicrographs of mesial sagittal sections of the basi-occipital bone. Vitamin A deficiency caused but little change on surfaces 3 and 4, so that the outer table of bone continued to grow normally and therefore to withdraw from the inner table, with the consequent enlargement of the marrow cavity and thickening of the bone.

The above facts apply to dogs killed when 20–30 weeks old, which, judged by their behaviour during life, had been depleted of vitamin A stores for 8 or more weeks. In older animals the rate of growth of the basi-occipital bone diminishes and the differences between the number and activity of osteoclasts and osteoblasts on surfaces 1 and 2 in +A and −A dogs, although still evident, are not as obvious as in the younger animals (see Pl. 3*a*, *b*, compared with Pl. 2*a*, *b*).

Drawings of coronal sections of the central portion of the basi-occipital bone of older +A and −A litter-mates, animals which were 33 weeks old at death, can be seen in Pl. 4. In the +A dog (Pl. 4*a*), which had 5000 i.u. vitamin A daily, the inner and outer tables of bone have fused to form a compact structure and the marrow cavity has been eliminated. In the −A dog (Pl. 4*b*) the bone is cancellous, with a large marrow space, and surface 1 is almost flat as compared with that of the +A animal, which is modelled to the natural shape of the brain stem. It will be seen that on this surface in the +A dog there are still abundant osteoclasts which are more numerous in the centre than at the sides, a fact which may account for the hollowing near the midline. In the −A dog, on the other hand, there are osteoblasts at work, but no active osteoclasts. Thus, so far as surface 1 is concerned, the absence of vitamin A, as in the younger animal previously described, has reversed the type of bone cell at work. Surface 2, however, of the older −A dog (Pl. 4*b*) is different from that of the younger animal (Pl. 2*b*) in that there are no active osteoclasts. It is indeed interesting that the fusion of the inner and outer tables of bone in the +A dog appears to be accompanied in the corresponding −A animal with the disappearance of all active bone cells, both osteoclasts and osteoblasts, from surfaces 2 and 3 (Pl. 4*b*). In the case of surface 4, there are only a few scattered active osteoclasts left in the +A animal (Pl. 4*a*), whereas in the −A there are no osteoclasts and only a few active osteoblasts laterally (Pl. 4*b*).

The basi-occipital bone of a litter-mate which was killed at about the same age, having received an A-deficient diet for 24 weeks of the experiment and, during the last 23 days of its life, vitamin A (5000 i.u. daily), is shown in the photomicrograph, Pl. 3*c*, and in the drawing, Pl. 4*c*. Though this dog is referred to as a recovery animal, it will be seen that the shape (Plate 4*c*) and texture (Pl. 3*c*) of the bone have not become normal; surface 1 is still flat and the bone cancellous. On the other hand, examination of the type and number of active bone cells on the different surfaces shows the great effort being made to bring about restoration of the bone to the normal shape. On surface 1 numerous active osteoclasts are apparently removing bone, while it is being laid down by the very active osteoblasts on surface 2, so that the inner table of bone is retreating to allow greater space for the brain stem (Pl. 4*c* and Pl. 3*c*). Surfaces 3 and 4 are also affected. Surface 3 shows osteoblastic activity, and on surface 4 active osteoclasts are seen, although there are many less than on surface 1.

It will be asked how soon after the addition of vitamin A to the diet the osteoclastic and osteoblastic activity returns to that seen in the young +A animal and whether the shape of the bones can ever be restored to normal. Insufficient evidence is as yet available to answer either of these queries fully, but it is known that 3 days after the addition of vitamin A to the diet cellular changes are negligible, but that after 13 days the osteoclasts and osteoblasts are rapidly correcting the abnormal growth of the period of vitamin A deficiency. This can be seen by comparing Pl. 5*a*, *b* and *c*. The three animals concerned were litter-mates. The +A and −A dogs were killed when 20 weeks old and from this time for 13 days the recovery animal was given 2000 i.u. vitamin A acetate daily. Pl. 5*a* is a photomicrograph of the basi-occipital bone (inner table) in the +A, Pl. 5*b*, in the −A, and Pl. 5*c* in the recovery animal. What the ultimate outcome of these changes in recovery animals will be, it is not yet possible to say. It would seem, however, that a continuation of the activity of the bone cells seen in Pl. 5*c* is calculated to bring about a return to the normal state both as regards the shape of the bone and the expansion of that part of the brain cavity bounded by the basi-occipital bone.

Labyrinthine capsule and VIII nerve

The bony labyrinth is probably fully formed in the young animal before the time of special feeding in these experiments begins (6–7 weeks). At this age also the periosteal bone covering the labyrinth on the side adjacent to the brain is nearly fully formed, as is indicated by the few and inactive bone cells on this surface. On the same surface of the periosteal bone of the labyrinth there are in +A animals when about 20 weeks old a few inactive osteoclasts and inactive osteoblasts, in contrast to the active osteoclasts found on surface 1 of the basi-occipital bone.

In an A-deficient animal of this age these osteoblasts become very active, so that, whereas in the case of the basi-occipital bone the change produced by A deficiency is mainly, although not entirely, due to a failure to absorb bone, the outstanding character of the same deficiency on the labyrinthine capsule is to produce excessive laying down of bone. As previously shown (Mellanby, 1938), parts of this capsule may in A-deficient animals become so thickened that it largely blocks the internal auditory meatus (Pl. 6, Fig. 1 *a*, *b*). The extra bone formed is, in the younger animals (Pl. 6, Fig. 2*a*, *b*), cancellous with large fatty marrow spaces. Fewer spaces are seen, however, as the animals grow older, until at 33 weeks of age (Pl. 7) the bone is relatively compact even after 26 or so weeks of the A-deficient diet. The marrow spaces of the younger, but rarely of the older, animals sometimes contain a few active osteoclasts.

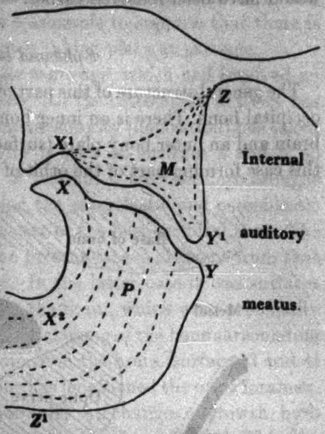
In the −A animals the enlarged periosteal bone adjacent to the brain increases the distance from the helix to the medulla, lengthens the internal auditory meatus and causes stretching of the VIII nerve. Within the internal auditory meatus there is a change in the position of bone cells reminiscent of that of the basi-occipital bone (Pl. 6, Fig. 2*a*, *b*), slight osteoclastic activity on the surface adjacent to the VIII nerve in the +A dog becoming in the −A animal osteoblastic activity with active osteoclasts on the marrow surface (equivalent to surface 2 of the basi-occipital bone). There is a definite deposition of bone within the meatus, which causes compression and, as it is not laid down equally but is found rather in nodules, tortuosity of the nerve.

Pl. 7*a*, *b* are drawings of the labyrinthine capsules of the +A and −A litter-mates which were 33 weeks old when killed. The increased thickness of the bone in the −A dog (Pl. 7*b*) is clear. There are practically no active osteoblasts or osteoclasts in either series of sections, thus indicating that both normal growth (Pl. 7*a*) and abnormal growth (Pl. 7*b*) have ceased. In Pl. 7*a* the few active osteoclasts seen are on the surface of the internal auditory meatus, which suggests that it is still necessary at this age to have a mechanism at work capable of modifying its size and shape.

If the absence of active osteoblasts and osteoclasts from the labyrinthine periosteal bone in the +A and −A animals of this experiment suggests absence of control by vitamin A, it is only an illusion. Proof of this can be seen in the labyrinthine capsule of the third litter-mate of the same age (Pl. 7*c* and Pl. 6, Fig. 2*c*), which was on a diet deficient in vitamin A for 24 weeks and then received for the last 3 weeks (23 days) of its life 5000 i.u. of vitamin A daily. The reaction on the addition of vitamin A is clearly tremendous, the surface of the periosteal bone being covered with active osteoclasts (Pl. 6, Fig. 2*c*) which are obviously there in an attempt to restore the bone to its normal (+A) shape (Pl. 7*a*).

There appears to be a definite plan in this absorption of excess bone, since great osteoclastic activity is found wherever osteoblasts have been active during the period of A deficiency. Thus the whole surface of the periosteal bone adjacent to the brain and within the internal auditory meatus is covered with active osteoclasts in the recovery animal (Pl. 7*c*). Since all this periosteal bone overgrowth is due to the excessive activity of osteoblasts during the −A period, this result is in keeping with the general recovery reaction. One point of special interest is seen in the recovery animal, namely, the presence of abundant osteoclasts on a surface marked X^1–Y^1 in the sketch (Text-fig. 2), and the almost complete absence of these cells from the adjoining surface X–Y. Although the portion of bone marked M is of periosteal origin, like that marked P, it seems probable that the mode of growth of M is rather different from that of P. A possible difference is suggested in the sketch, and it will be seen that M may grow in two directions, Z–Y^1 and X^1–Y^1, whereas P probably grows in one direction as bony layers deposited on surface X–X^2. This explanation of the method of bone growth during the −A period (Pl. 7*b*) would allow the appearance of osteoblasts on surface X^1–Y^1 of the recovery animal (Pl. 7*c*) to be regarded as in line with the usual recovery reaction in regard to osteoclasts.

It seems legitimate to assume from this experiment that the apparent absence of active osteoclasts and osteoblasts on the normal labyrinthine capsule at this age does not mean that vitamin A has ceased to control, directly or indirectly, either the bones or the bone cells, but that it holds a guiding hand on these cells so that they produce and mould periosteal bone covering the labyrinth only in amounts and shape compatible with the easy passage of the VIII nerve from the labyrinth to the central nervous system. If, during the absence of the vitamin, the periosteal bone grows so that excessive bone is laid down, then the return of the vitamin to the body not only reverses the process but causes an exaggerated response of bone cells calculated to clear away the excessive bone formation and bring its shape quickly back as near to the normal as possible.

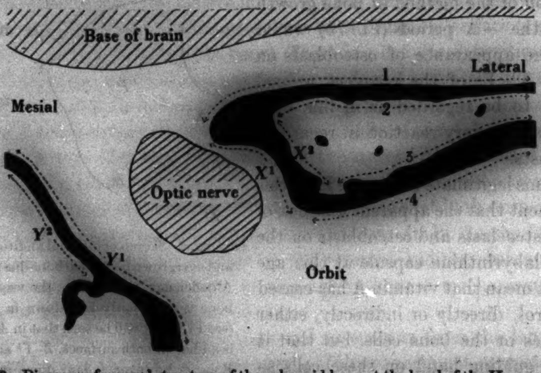


Text-fig. 2. Sketch of internal auditory meatus and overgrown periosteal bone due to vitamin A deficiency, to illustrate the way in which bone is apparently laid down in M and P (see Pl. 7). It will be seen that in M the bone is added on both surfaces, Z–Y^1 and Y^1–X^1. Bone P, however, is laid down in the lines indicated, and the surface of growing bone faces in one direction only. It is usual in −A+A animals to see osteoclastic action where, in the −A, osteoblastic action has been predominant. Thus surfaces Z–Y^1, Y^1–X^1 and Y–Z^1 would be expected to show much osteoclastic activity and surface Y–X to show little or none.

Comparing the reactions of the labyrinthine capsule and the basi-occipital bone in A deficiency, it is seen that the first is an example of excessive bone formation following the loss of vitamin A, the return of the vitamin causing removal of the hyperplastic new bone. In the second, loss of vitamin A is followed primarily by cessation of removal of bone already laid down, although in the earlier weeks of the experiment there may be in addition a small deposition of new bone; on the return of the vitamin the bone removed by the hosts of active osteoclasts is for the most part bone which, under normal conditions, would have been removed earlier.

Sphenoid bone and II nerve

The general structure of this part of the sphenoid is similar to that of the basi-occipital bone. There is an inner bony plate (surfaces 1 and 2) adjacent to the brain and an outer bony plate (surfaces 3 and 4) remote from the brain, but in this case forming part of the orbit of the eye. Text-fig. 3 shows this formation.



Text-fig. 3. Diagram of general structure of the sphenoid bone at the level of the II nerve foramen. Surfaces have again been numbered to simplify description in text. Surface 1 is the bone surface adjacent to the brain stem. Surface 2 is the marrow surface of the same inner table of bone. Surface 3 is the marrow surface of the outer table of bone. Surface 4 is the outer surface of the outer table of bone. Surface X^1 is the lateral wall of the II nerve foramen and joins surfaces 1 and 4. Similarly, X^2 is the marrow surface joining surfaces 2 and 3. Surface Y^1 is the mesial wall of the II nerve foramen and Y^2 the marrow surface of the same plate of bone.

The sections to be described are coronal through the centre of the optic foramen, at right angles to the long axis of the head. They therefore show the mesial and lateral bone of the foramen wall, but not the anterior or posterior parts. For ease in description, the bone of the lateral wall of the optic foramen has been divided into surfaces X^1 (adjacent to the second nerve and connecting surfaces 1 and 4) and X^2 (the marrow surface connecting surfaces 2 and 3). The mesial wall has been similarly divided into surfaces Y^1 and Y^2. Pl. 8*a* is a drawing of the optic foramen and sphenoid bone in a +A dog aged 33 weeks. It will be seen that there is no osteoclastic activity on surfaces 2, 3, Y^1 and X^2, a little on surfaces 1, 4 and Y^2, with rather more on surface X^1. Active osteoblasts are confined to surfaces Y^1 and X^2. This would suggest that there is very little growth and moulding occurring at this age, with the possible exception of a little on the walls of the foramen. From the positions of the osteoblasts on surfaces Y^1 and X^2 and the osteoclasts on Y^2 and X^1, it is reasonable to suppose that there is still a small lateral movement of the foramen taking place at this age.

Pl. 8*b* is a drawing of a litter-mate of the same age, which had received an A-deficient diet for 26 weeks. It will be seen that the osteoclastic activity has ceased on surfaces 1 and 4 and that osteoblastic activity (deposition) has taken its place on surface 4. Osteoclastic activity is seen on surfaces 2 and 3, so enlarging the marrow space. The absence of vitamin A has therefore brought about a thickening of the bone by reversing the osteoclastic and osteoblastic activity, as has been seen to happen in the case of the basi-occipital bone. The actual mechanism of bone thickening in the present case is different from that described above in the basi-occipital bone. In the latter case it was surfaces 1 and 2, forming the bony plate adjacent to the brain, which were principally affected by the absence of vitamin A. In the thickening of the bone surrounding the optic foramen it is the bony plate remote from the brain (surfaces 3 and 4) which is affected. This thickening of the bone has lengthened the optic foramen. What, meanwhile, has been happening to its wall? All changes of growth, both absorption and deposition, seem to have ceased on surfaces X^1 and X^2 in the −A animal, while growth processes have continued in a normal way on the lengthened surfaces Y^1 and Y^2. This would have the effect of narrowing the foramen, for, while the mesial surface would move laterally, the lateral surface would remain stationary, thereby constricting the nerve and vessels passing through the foramen.

If we now examine the recovery animal (Pl. 8*c*), a third litter-mate which was maintained on an A-deficient diet from the age of 7 to 31 weeks and then received 5000 i.u. of vitamin A daily for 3 weeks (23 days), we see that there is a return to the normal as regards position of active cells. Surfaces Y^1 and Y^2 again show no variation in type of cell, although there may be some increase in intensity of growth. Surfaces 1 and 2, which showed comparatively little change due to A deficiency, also show but little change on the return of the vitamin. The greatest response to the addition of the vitamin is seen on surfaces 3, 4, X^1 and X^2. There is obviously a great eating away of bone from surfaces 4 and X^1, with a correspondingly great deposition on surfaces 3 and X^2. It seems obvious that continuation of these processes will restore the foramen to the normal (+A) diameter, reduce its length by reducing the thickness of the sphenoid bone, and correct its position by moving it laterally.

Notes on other bones

Of the bones examined, most appear to react in the same way as the basi-occipital, but there are several special regions in which strong osteoclastic activity in the +A becomes reduced osteoclastic activity in the −A, complete cessation of absorption and deposition of bone being uncommon. One of these positions is part of the surface of the vertebral column adjacent to the lateral side of the posterior root ganglion. It was shown in the Croonian Lecture that osteoclasts may remain in this position in the −A animal, and further examinations have confirmed that these cells persist here in a comparatively inactive state when they have completely disappeared from the rest of the surface adjacent to the spinal cord. Reversal from osteoclastic to osteoblastic activity does, however, occur in very severe A deficiency. Recovery animals show strong osteoclastic activity over the whole of the surface of the vertebrae adjacent to the nervous system, including this special region, irrespective of whether the deficiency has been severe or only of a degree to produce reduced absorption.

Bones in which efforts are made by bone cells to correct the abnormal shape, when vitamin A is added to the diet of an animal showing signs of A deficiency, include the long bones (femur and radius), the pelvis, the petrous ridge and the walls of the V nerve foramen. Osteoclastic action to reduce the enlarged petrous ridge and to widen the V nerve foramen is particularly intense.

DISCUSSION

The foregoing account of the effect on bone of vitamin A, its absence and its replacement in the diet of an A-deficient animal, shows clearly that it plays an important part in controlling bone growth and shape, its special function being to model bones. In the case of bones adjacent to the growing nervous system, even the finer degrees of moulding have to be specially adjusted. Thus, the precise chemical control of bone development in this position is of the utmost importance to the animal's well-being. As the nervous system and its branches grow, the brain and cord cavities and the foramina which allow the passage of nerves to and from the central nervous system must enlarge to accommodate these tissues. If vitamin A is deficient in the growing puppy, bone growth is so changed that the skull cavity and the spinal canal enlarge inadequately and irregularly, and the foramina may even become smaller, with disastrous results, which may include destruction of the olfactory, optic, trigeminal and auditory nerves and the posterior roots of the spinal cord.

If, after a period of some weeks, the vitamin is restored to the defective diet, there is a vigorous attempt to correct the wrong growth of the deficient period. This fact strengthens the evidence that vitamin A exerts a powerful controlling influence on the elements responsible for co-ordinated bone growth and thereby brings about the necessary adjustment between the growth of bone and nervous tissue. Whether this effect of the vitamin on bone is direct or indirect is not known, nor is it known to what extent the effect extends beyond the period of active growth in early life. Evidence has been given to show, however, that the vitamin's influence on a bone may be present when that particular bone has apparently ceased to grow, e.g. the labyrinthine periosteal bone, although other bones are growing vigorously at the time. Also it is known that in adult dogs the removal of vitamin A from the body causes a very slow overgrowth of the periosteal bone of the labyrinth.

It may be added that bones remote from central nervous tissue, such as the long bones and pelvic bones, are also affected by vitamin A, the effect of its absence being loss of the finer moulding and the production of a coarser general appearance. What such a change in shape means in terms of the physiology of the long bones and their adjoining tissues cannot be discussed here, but it is certainly not so serious as the destruction or other pathological change in nervous tissue that follows the dysplasia of skull and vertebral bones. The pelvis of reduced internal dimensions produced by vitamin A deficiency, although unlikely to prove as important in parturition as that produced by vitamin D deficiency, may also be of some practical significance, but this is also a subject which, it is hoped, will receive further attention later.

Although the general result of these changes on bone growth and shape is obvious, the problem becomes more difficult when an attempt is made to generalize in terms of the reactions of osteoclasts and osteoblasts to vitamin A. One of the difficulties is the variation in intensity and manner of bone growth at different ages. In, for instance, the basi-occipital bone of a +A puppy aged 20 weeks, bone cell activity is greater than in an animal on the same diet aged 30 weeks. Correspondingly the abnormal cell activity in −A animals will be greater at 20 than at 30 weeks of age. If, however, vitamin A is added to the diet of a deficient animal when 30 weeks old, the bone cell activity returns to the normal type, but the intensity is greater than that seen in the normal 20-week +A animal, and therefore much greater than in the normal +A animal of 30 weeks. Then there is the further difficulty that the mode of bone growth in different parts of the skeleton varies. For instance, in the basi-occipital bone there is normally absorption of bone on surface 1 and deposition on the marrow surface (surface 2) whilst at the same time there is in the femur deposition on the periosteal or outer surface and absorption on the marrow surface. This is an extreme example, but these differences and others of a minor degree in osteoclastic and osteoblastic activity frequently occur, especially in bones of complicated structure. By examining this abnormal bone growth and perhaps more easily by noting the efforts to correct it that follow the addition of vitamin A to the deficient diet, the mechanism of normal growth and the answer to the question as to how vitamin A controls bone shape and growth at any point will become clear.

It might be asked why it should be assumed that the reactions of bones to vitamin A deficiency and to the return of this vitamin indicate the normal way in which a particular bone grows. The answer is that in the case of bones adjacent to nervous tissue and especially near the central nervous system, the absence of vitamin A in most cases results in bone dysplasia, which either destroys or threatens to destroy local nervous tissue. The bone change which follows the restoration of vitamin A to the body is in the direction of a return to normal shape or at least to a shape which brings relief of pressure to the nervous tissue. In such cases, therefore, it seems legitimate to assume that the recovery reaction of bone indicates an exaggerated form of the normal growth process. So far as is known, the only cells controlling bone growth are osteoblasts and osteoclasts, but it has been seen above that nature uses these two types of cells as regards number and degree of activity in different ways at different points of growth. In the examples given above, it is clear that the normal manner of growth, that is to say the growth which takes place in the presence of vitamin A, varies in each case. The salient facts as regards bone cell activity during growth in these positions are as follows:

Basi-occipital bone

Normally, there is removal of bone from the surface 1 adjacent to the brain stem by osteoclasts and a laying down of bone on the corresponding marrow surface 2. Deficiency of vitamin A reverses these processes and the growth in depth of the posterior fossa is reduced. Recovery changes produced by the addition of vitamin A to the diet again reverse the type of cell activity.

Labyrinthine capsule

Normally, after the early laying down of the periosteal bone covering the labyrinth, the osteoblasts are inactive and the few osteoclasts remaining active are found on the surface of the internal auditory meatus, possibly to enlarge the meatus to allow easy passage of the VIII nerve. Deficiency of vitamin A causes a great laying down of bone, which partially blocks the meatus and injures or even kills the nerve. Recovery changes which follow the addition of vitamin A are directed to the removal by osteoclasts of this superfluous bone.

Optic foramen and the II nerve

In normal growth the foramina move laterally away from the middle line and each other. In this movement the osteoblasts and osteoclasts work in a co-ordinated way so as to keep each foramen open and possibly to enlarge it for the safe passage of the growing optic nerve. When vitamin A is absent from the body, this lateral movement stops or is reduced, and the foramina are smaller in diameter and the optic nerves are squeezed. Addition of the vitamin to the diet of a deficient animal brings the osteoclasts and osteoblasts into a state of hyperactivity, so as to correct the damage done during the period of deficiency.

Here then we have three instances of growth of bone adjacent to the nervous system where, although vitamin A deficiency and recovery therefrom have the same general results, disposition of the bone cells is different in each case.

The following summary of changes due to the presence and absence of vitamin A probably applies to all the growing bones described above.

(1) Neither in the presence nor in the absence of vitamin A are active osteoblasts and active osteoclasts intimately mixed up at any one part of a surface of a table of bone, although the activity of each may be much greater at one part of a surface than at another. For instance, the place where a muscle is inserted into a bone surface is more likely to have a greater number of osteoclasts than another point of the same surface where no muscle is inserted. There are rare occasions also when one portion of a surface shows the laying down of bone and another part of the same surface shows bone removal. When this happens the parts affected are distinct (see Pl. 8*b*, surface 3).

(2) When osteoclastic activity is evident on one surface, the opposing surface of the same piece of bone is usually covered with active osteoblasts. This type of co-ordination is clearly necessary, since otherwise the bone would disappear and the marrow would be exposed. This does happen occasionally in recovery cases over very small areas, but they are not sufficiently large to weaken the bone structure. By this means the basi-occipital bone is moved in the direction away from the base of the brain, thereby allowing expansion of the posterior fossa. Similarly, it is the method by which the optic foramina are moved away from the middle line during normal growth or during recovery from vitamin A deficiency.

(3) The effect of removing vitamin A from the animal body is usually to stop osteoclastic activity on the most effective surface, for example, in the basi-occipital bone, the surface near the nervous tissue (Text-fig. 1, surface 1). If the osteoclastic action has been great in the presence of vitamin A, then the removal of the latter usually results in cessation of activity at that surface or a weak osteoblastic action. If the osteoclastic action at the time the deficiency is established is slight, then the removal of vitamin A is more likely to be replaced by osteoblastic activity and a vigorous laying down of bone on that surface.

(4) The recovery changes in dysplastic bone following the restoration of vitamin A to a diet deficient in this substance are always vigorous, much more so in fact than the original growth processes in the presence of vitamin A. The cells—osteoclasts and osteoblasts—are restored to their normal positions but are greatly increased in number and activity.

(5) Although the absence of vitamin A during bone growth may cause devastating effects on the nervous system, its removal from the diet is not synonymous either with cessation of bone cell activity or with anarchy of

these cells. The natural process of osteoclastic and osteoblastic activity at each place seems to be pre-determined and, the orders having been given, vitamin A sees that they are carried out. If the vitamin is absent, growth goes awry, since the orders are not only disobeyed but reversed or changed in a regular way. Similarly, the recovery process which follows the restoration of vitamin A involves a return of cell activity to the normal positions but with increased activity, although, as we have seen above, the normal cell type varies from place to place.

Wolbach & Bessey (1941) confirmed in rats the destruction of nervous tissue by bone pressure produced by vitamin A-deficient diets (Mellanby, 1938, 1941), but their interpretation of the cause of the bone pressure was different. On their deficient diets they found that bone growth was retarded or stopped, but that the nervous system continued to grow at a normal rate and so became compressed. The explanation is not so simple, although recent evidence (Mellanby, 1944) showing the relative importance of osteoclastic activity in normal growth makes it easy to understand how cessation or retardation of bone growth might appear to be a satisfactory explanation, especially in short-term experiments. The prime factor in bone growth, however, must always be a laying down of bony tissue by osteoblasts, and this activity is not stopped by vitamin A deficiency although its place of action may be altered.

In the experience of this laboratory the particular vitamin A-deficient diets used in rat experiments have allowed good growth of bone and the rats have developed a bone dysplasia (Mellanby, 1938) more slowly than the animals described by Wolbach & Bessey. As in the puppies, this bone dysplasia, which causes compression of the nervous system, is the result of bone growth lacking the co-ordination found in normal growing bone. It is possible that variations in the basal diet, and thereby in the rate of depletion of vitamin A reserves, may account for the different results and interpretations in rat experiments. Clearly there is still a missing factor between the two investigations, and it is hoped that further experiments will bring them into agreement.

SUMMARY

1. The foregoing experimental evidence shows that the general function of vitamin A as regards growing bone is to control its shape and specially its fine moulding by influencing the position and the activity of osteoclasts and osteoblasts.

2. The addition of vitamin A to the diet of an A-deficient animal during growth brings about a return of osteoclastic and osteoblastic activity to the surfaces where it is normally found. This reaction is often of a very intense nature, and its object is clearly to restore the normal shape of the dysplastic bones formed during the deficient period. The present experiments did not continue long enough to establish whether this object is ever fully attained.

3. The change in position of activity and in number of osteoclasts and osteoblasts resulting from vitamin A deficiency is orderly, not anarchic, and is usually a modification or even a reversal at the effective surfaces, deposition of bone taking place where there was previously either absorption or no bone cell activity.

4. Although vitamin A deficiency causes a general thickening and dysplasia of bone by its effect on osteoclasts and osteoblasts, the method of producing this abnormal state varies, as is shown in the three regions discussed, namely the basi-occipital, the periosteal bone covering the labyrinth, and the sphenoid bone in relation to the optic foramen. The recovery changes on adding vitamin A are also different in each case.

5. The altered shape of bones adjacent to the nervous system may destroy cranial and spinal nerves and exert other harmful effects, but the recovery changes in dysplastic bone on the addition of vitamin A to the diet take place independently of the condition of the adjacent nerve or nervous tissue.

I wish to thank the staff of this laboratory for their important part in this investigation and especially to express my appreciation of the skilful help given by Mr R. J. C. Stewart.

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EXPLANATION OF PLATES

In the drawings of Pls. 4, 7 and 8 active osteoclasts are indicated by black dots and active osteoblasts by black lines; inactive cells of either type are not marked.

Plate 1

Photomicrographs of sections through basi-occipital bones to show inactive and active osteoblasts (× 437) and osteoclasts (× 328), as referred to in text. (*a*) Inactive osteoblasts—cells flattened on bone surface. (*b*) Active osteoblasts—cells cubical on bone surface. (*c*) Inactive osteoclasts—cells lying flattened between bone surface and periosteum; very little evidence of bone absorption. (*d*) Active osteoclasts—multinucleated cells prominent between bone surface and periosteum; note lacunae due to absorption. (*e*) One inactive osteoclast (× 875). (*f*) One active osteoclast (× 875).

Plate 2

Photomicrographs of sagittal sections (×130) of the inner table of the basi-occipital bones of +A (*a*) and −A (*b*) litter-mate dogs to show reversal of osteoclastic and osteoblastic activity on surfaces 1 and 2. Duration of experiment 19 weeks and final age 25 weeks. (*a*) *+A diet*: Surface 1, many active osteoclasts, no active osteoblasts. Surface 2, many active osteoblasts, no active osteoclasts. (*b*) *−A diet*: Surface 1, many active osteoblasts, no active osteoclasts. Surface 2, many active osteoclasts, no active osteoblasts.

Plate 3

Photomicrographs of coronal sections (×114) of the inner table of basi-occipital bones of three litter-mate dogs, +A (*a*), −A (*b*) and recovery or −A followed by +A (*c*). Duration of experiment 26–27 weeks and final age 33–34 weeks. The effect of removing vitamin A from the diet is seen by comparing (*a*) with (*b*) and of adding vitamin A to an A-deficient diet by comparing (*c*) with (*b*). (*a*) *+A diet*: Surface 1, some slight osteoclastic activity. Surface 2, inactive osteoblasts, (*b*) *−A diet*: Surface 1, some slight osteoblastic activity. Surface 2, inactive osteoblasts. (*c*) *Recovery or −A followed by +A diet*: Surface 1, great osteoclastic activity. Surface 2, very strong osteoblastic activity. *Note.* The osteoclasts and osteoblasts have again become active in their normal positions.

Plate 4

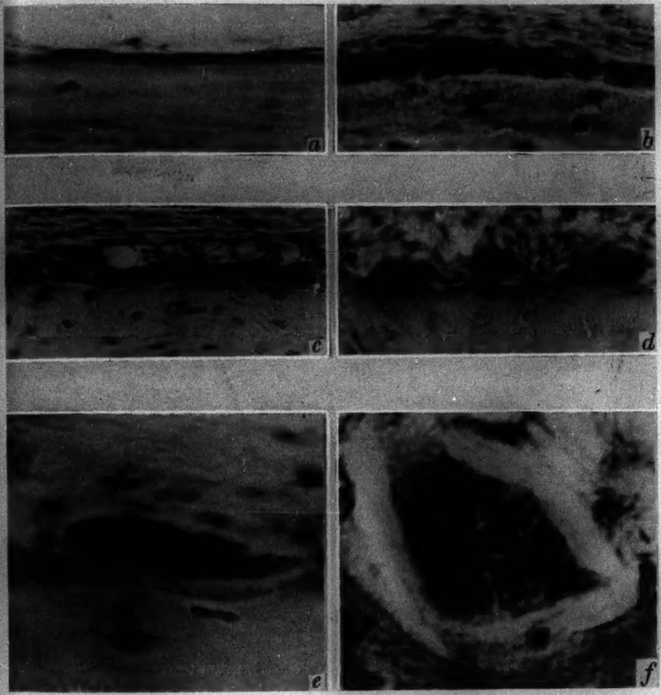
Drawings of coronal sections through basi-occipital bones of three litter-mate dogs, +A (*a*), −A (*b*) and −A followed by +A (*c*). Duration of experiment 26–27 weeks and final age 33–34 weeks. (*a*) *+A diet*: Inner and outer bone tables have fused, with elimination of surfaces 2 and 3. Surface 1, abundant active osteoclasts, especially towards middle line. Surface 4, a few scattered osteoclasts. (*b*) *−A diet*: Bone still cancellous, with large marrow cavity. Surface 1, some osteoblasts (reversal from same surface in (*a*)). Surface 2, no cell activity. Surface 3, no cell activity. Surface 4, a few active osteoblasts but otherwise no activity. (*c*) *Recovery or −A followed by +A diet* (+A diet for last 3 weeks (23 days)): Surface 1, osteoclastic activity like (*a*) but much greater. Surface 2, much osteoblastic activity. Surface 3, some osteoblastic activity. Surface 4, a little osteoclastic activity, especially at periphery. *Note.* Reversal of action in (*c*) brought about by added vitamin A, especially on surfaces 1 and 2.

Plate 5

Photomicrographs of sagittal sections (×114) of inner tables of basi-occipital bones of three litter-mate dogs, +A (*a*), −A (*b*) and −A followed by +A (*c*). Duration of experiment 14–16 weeks and final age 20–22 weeks. Dog (*c*) was on −A diet for 14 weeks and +A diet for 2 weeks (i.e. 13 days' recovery period). (*a*) *+A diet*: Surface 1, a few osteoclasts. Surface 2, many active osteoblasts. (*b*) *−A diet*: Surface 1, many active osteoblasts. Surface 2, some osteoclasts. (*c*) *−A followed by +A diet*: Surface 1, many active osteoclasts. Surface 2, many active osteoblasts. *Note.* There has been a reversal of cell activity at surfaces 1 and 2 when vitamin A was removed from the diet (compare (*b*) with (*a*)). On adding vitamin A to a deficient animal (*c*), the cells have again reverted to their normal position (*a*), but are much more numerous and very active.

THE JOURNAL OF PHYSIOLOGY, Vol. 105, No. 4

PLATE 1



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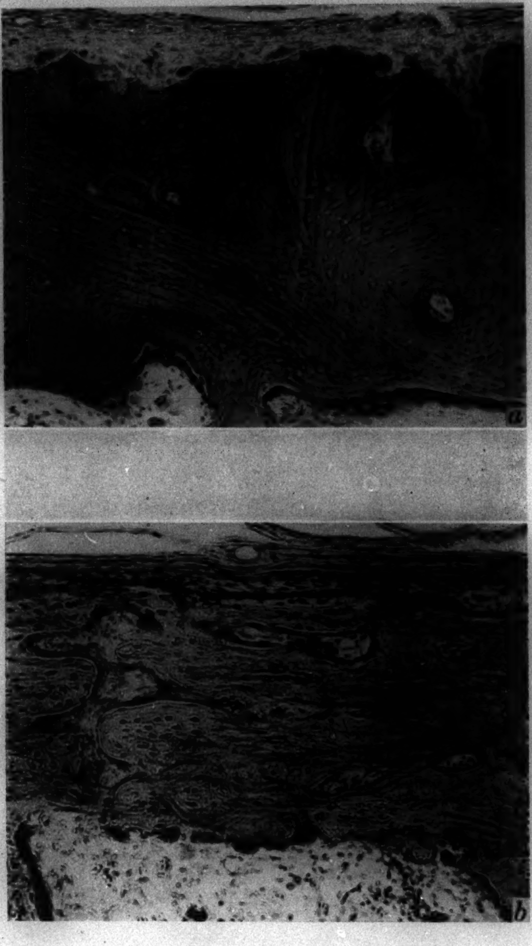
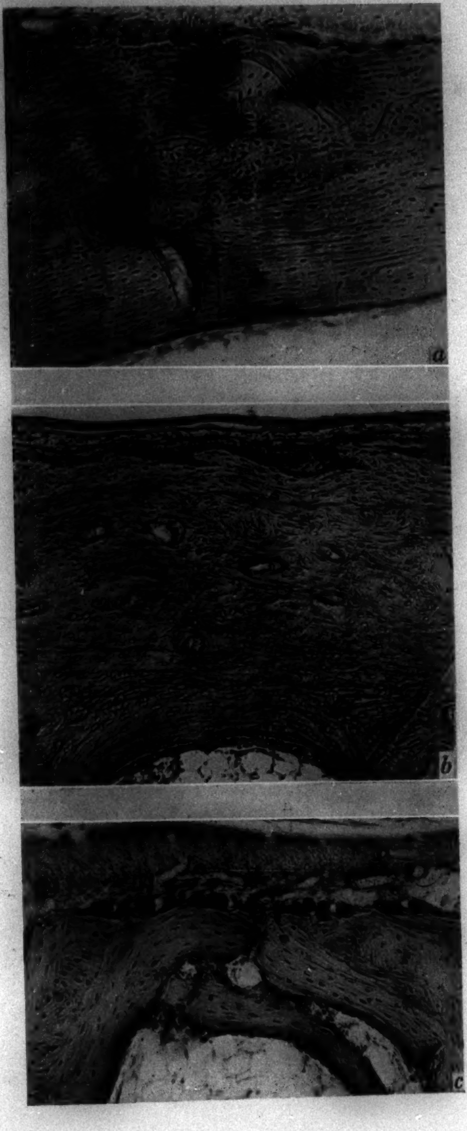
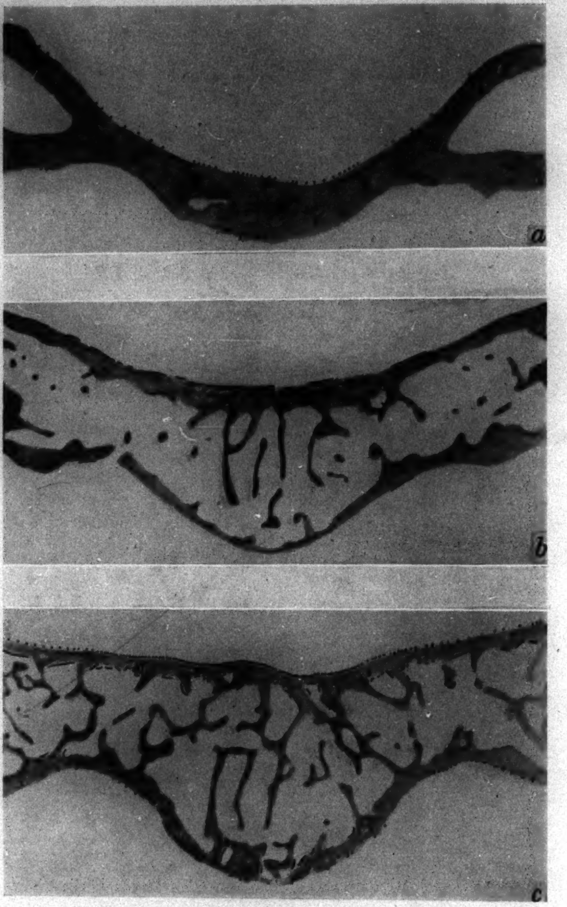

a
b

PLATE 3

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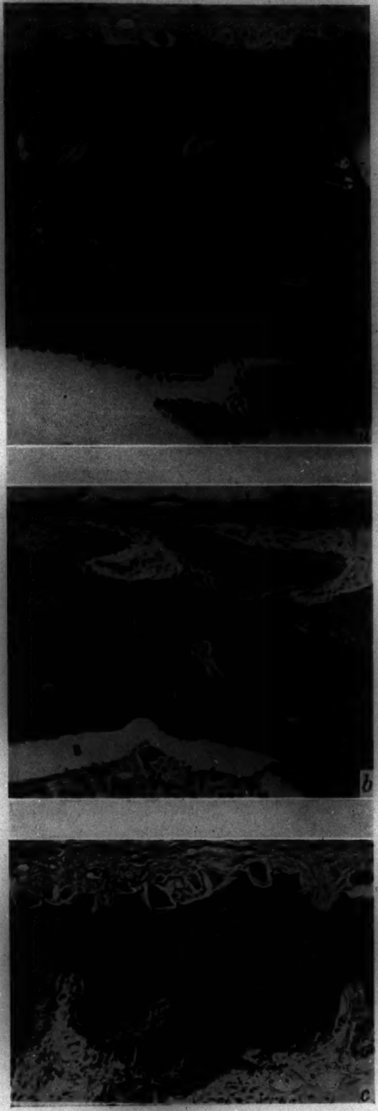
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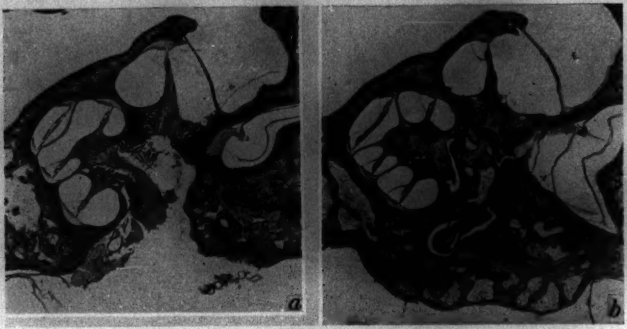


Fig. 1.

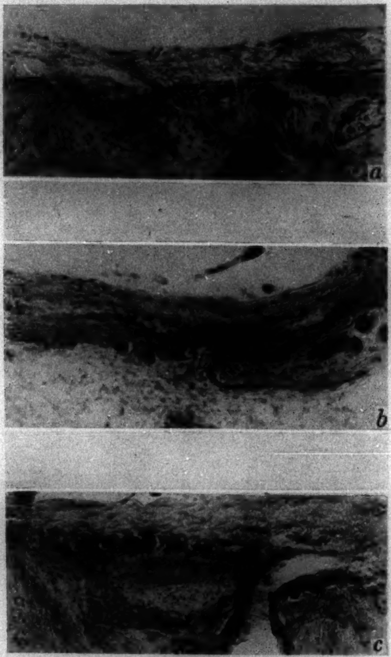
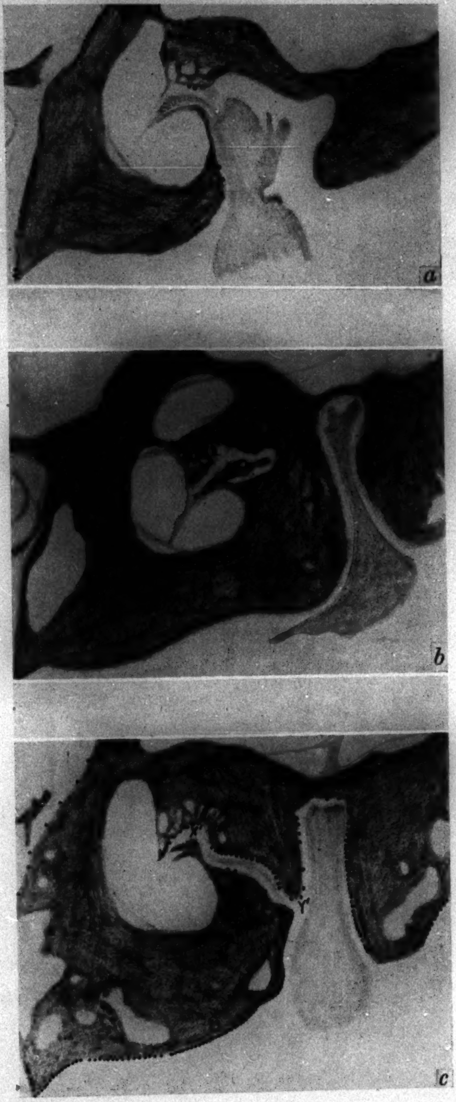


Fig. 2.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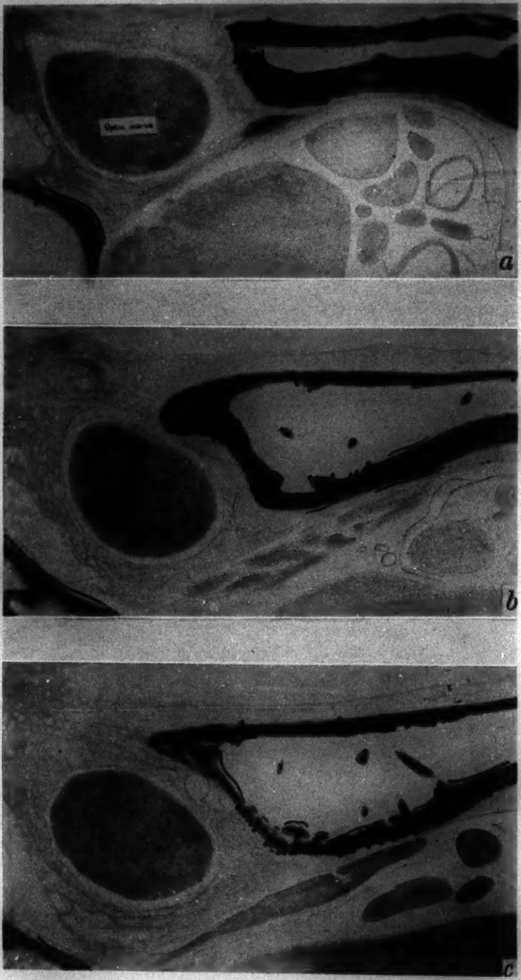
Optic nerve
a
b
c

Plate 6

Fig. 1. Photomicrographs (× 6·1) of the labyrinths of two litter-mate dogs, +A (*a*) and −A (*b*). Duration of experiment 22 weeks and final age 29 weeks. *Note* the laying down of much excess periosteal bone in (*b*). The internal auditory meatus appears to be occluded, but this is due to its tortuous course, and although it is much constricted the meatus is patent, as can be seen by examining serial sections. The VIII nerve is twisted, compressed and lengthened.

Fig. 2. Photomicrographs (× 114) of the bone adjacent to the internal auditory meatus in three dogs, +A (*a*), −A (*b*) and −A followed by +A (*c*): (*a*) *+A diet*: Duration of experiment 18 weeks and final age 27 weeks. Some osteoclastic activity on the bone surface adjacent to the VIII nerve. (*b*) *−A diet*: Duration of experiment 18 weeks and final age 27 weeks. Osteoblastic activity on the surface adjacent to the VIII nerve and osteoclasts now seen on the marrow surface. *Note*. Reversal of cell activity similar to that seen in the basi-occipital bones. (*c*) *−A followed by +A diet*: 24 weeks on −A diet followed by 3 weeks (23 days) on +A diet, and final age 34 weeks. Large number of active osteoclasts on the surface adjacent to the VIII nerve. *Note*. Cell activity has returned to the normal position, but is increased when compared with (*a*).

Plate 7

Drawings of coronal sections of labyrinths of three litter-mate dogs, +A (*a*), −A (*b*) and −A followed by +A (*c*). Duration of experiment 26–27 weeks and final age 33–34 weeks. During the last 3 weeks of the experiment (23 days), (*c*) received 5000 i.u. of vitamin A daily. (*a*) *+A diet*: Practically no active bone cells visible except a few osteoclasts on inner surface of internal auditory meatus. (*b*) *−A diet*: No active cells visible. In the internal auditory meatus there has been a great formation of new periosteal bone, which has squeezed the VIII nerve: the increased activity of osteoblasts resulting in the overgrowth has ceased. (*c*) *−A followed by +A diet*: The addition of vitamin A has brought about a tremendous reaction and active osteoclasts in large numbers have returned to surface 1 with the object of removing the superfluous bone. *Note* the layer of osteoclasts on the surface X^1–Y^1.

Plate 8

Drawings of coronal sections through the sphenoid bones of three litter-mate dogs, +A (*a*), −A (*b*), and −A followed by +A (*c*). Duration of experiment 26–27 weeks and final age 33–34 weeks. (See Text-fig. 3 for numbering of surfaces.) (*a*) *+A diet*: Very little cell activity on surfaces 1, 2, 3 and 4. Osteoclasts are seen on surfaces Y^2 and X^1, osteoblasts on surfaces Y^1 and X^2. (*b*) *−A diet*: No changes in cell activity on surfaces Y^1 and Y^2. Cessation of activity on X^1 and X^2. Surface 3 shows both osteoclastic and osteoblastic activity, and surfaces 1, 2 and 4 show reversal of cell activity. (*c*) *−A followed by +A diet*: No changes on surfaces Y^1 and Y^2. Activity on surfaces X^1 and X^2 has returned to the normal type, with an increase in intensity. Surfaces 3 and 4 show a great activity of bone cells and a reversal to the normal type; surfaces 1 and 2 show little change.

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The ratios of iron to oxygen, iron to colour and oxygen to colour in the blood of men and women. By Q. H. Gibson and D. C. Harrison.

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In a recent communication (Gibson & Harrison, 1945), we described the preparation of an artificial standard for use in haemoglobinometry by the alkaline haematin method. A haemoglobin value was assigned to this standard by iron and oxygen-capacity determinations on a series of samples of male blood. As Macfarlane & O'Brien (1944) obtained results suggesting that the oxygen to iron ratio is different in male and female bloods, it seemed possible that the ratios of iron to colour and oxygen to colour might also be different in the two sexes, and that therefore the colour standard would have different values when applied to men and to women. We therefore carried out two parallel series of determinations on ten male and ten female bloods taking precautions to avoid systematic errors affecting the sexes unequally. We found that the ratio of oxygen to iron was the same in both sexes. In male blood the value was 393 ml. oxygen/g. iron (S.D. mean 1·1 ml.); the corresponding figure for female blood was 393 ml. (S.D. mean 1·2 ml.). Colorimetric determinations made in a Spekker absorptiometer (with Chance OGr. 1 filter) on blood converted to alkaline haematin showed that the mean values for the ratios iron to colour and oxygen to colour differed by only 1 part in 400 between the two sexes. This difference was not significant. (Taking all the bloods together, the coefficients of variation for the ratios iron to colour and oxygen to colour were 1·0 and 1·2% respectively.) We conclude that there is no difference between the blood of males and females with regard to the properties investigated and that it is justifiable to use the same haemoglobin value for the standard in the colorimetric determination of haemoglobin in the blood of either sex.

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Visual acuity of the eye using lights of different colour. By H. Hartridge. *Department of Physiology, St Bartholomew's Hospital, London*

In 1920 the author advanced the view that the acuity of the eye depends on he perception by the photo-receptors of the retina of small differences of brightness. The differences of brightness varied in amount, being less in the case of absorption bands for which the difference of brightness between one photo-receptor and its next-door neighbour was about ½%.

In 1937 Shlaer criticized this hypothesis, substituting 'that the limiting factor in the resolution of the eye for the grating is the diameter of the pupil when it is less than 2·3 mm. and the size of the central cones when the pupil is larger than that'.

This hypothesis does not fit in either with the experimental results obtained by Shlaer, Smith & Chase (1942) or with those that the author has obtained recently. If Shlaer's hypothesis were correct, the acuity of the eye should improve as the wave-length of the light employed for illumination is made shorter. Shlaer's results with the grating show a very slight improvement as the wave-length of the light changes from 670 to 535. This is followed by a deterioration when the wave-length of the light is made shorter to 450. Using the Landolt's C test the acuity of the eye was found by Shlaer, Smith & Chase to be approximately constant at all wave-lengths. My experiments were done both with letters and with the C test. In none of these experiments was there any improvement when light of short wave-length was substituted for light of long.

Since Shlaer's hypothesis is shown to be unsound, the author's hypothesis replaces it, namely, that acuity depends on the perception of differences of light intensity by photo-receptors of the eye. The fact that the acuity is nearly the same for white, red, green and blue lights, indicates that in each of these cases the photo-receptors must be equally close to one another. If the photo-receptors of different colour response were distributed in a uniform manner, such would not be the case, neither would the high acuity of the eye be accounted for. This high acuity is explicable by supposing that there are in the retina collections of photo-receptors of one particular response, such as are required by the cluster hypothesis.

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The repair of the retinal image. By H. Hartridge.

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It has been shown in previous communications that the defects produced by the chromatic aberration of the eye are repaired by the anti-chromatic response. It is also known that defects in definition introduced by the lens system of the eye are largely, if not entirely, made good by simultaneous contrast. These processes of repair appear to be but examples of much more widely spread processes occurring everywhere throughout the retina. Thus even when monocular vision is being employed the presence of the blind spot does not ordinarily show itself. For example, if the centre of a large printed page be fixed, the lines of type will be seen to run continuously to right and left until the margin of the paper is reached. The lines continue over the blind spot as if it was not there at all. Now we have ample evidence for the statement that the blind spot is in fact completely devoid either of photo-receptors or of sensations. Thus there must be a complete break in the continuity of the response of the retina at this point.

The fact that this break is not observed in ordinary vision must be due to a process of extension, extrapolation, or repair in some part of the visual apparatus.

Experiments show that this process of repair is of a very detailed character. If a uniform red field is looked at, the blind spot is filled up with apparent red sensations, and similarly for other colours. Simple patterns such as the printed page mentioned above are reproduced at the blind spot apparently as easily as colours are.

This process of repair must play a highly important part in normal vision at parts of the retina which are not occupied by the blind spot. Because, according to the trichromatic theory of Young, or its modern development the polychromatic theory of Granit, photo-receptors responding to light of one colour are inactive to others. In consequence when the eye looks at a field which is lit by a monochromatic light, a large portion of that field would be occupied by small punctate scotomata which are produced by the inactive retinal receptors. But such scotomata are unobservable in normal vision, and this must be due to a process of repair which effaces them, just as it does the large scotomata produced by the papilla of the optic nerve.

Micro-stimulation of the human retina. By H. Hartridge.

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When the chromatic difference of magnification of the human eye has been corrected by suitable lenses, it has been found possible, by preserving the most perfect fixation, and by using extremely small test disks (micro-disks), to make a provisional plan of the photo-receptors near the fixation point of a human eye.

(1) The fixation points for red, green and blue lights are found to form a triangle with green at the apex, red at the right and blue at the left.

(2) An examination of the retinal astigmatism of the same eye, using green light, disclosed good acuity in vertical and horizontal directions, but not such good acuity in oblique directions. From these facts it was deduced that the area of maximum acuity for green rays is T-shaped, L- shaped or +-shaped. That is, that it has good vertical and horizontal components, but poor oblique ones.

(3) Using a small green fixation point and placing a grey micro-disk close to it in different positions, it was found that the grey disk varied in colour as follows:

Vertical	Grey	Down	Pale green
Up and right	Pink	Down and left	Pink
Right	Pale green	Left	Pale green
Down and right	Grey	Up and left	Pink

It was suspected where grey was perceived that this would have been blue if it had not been for the operation of the anti-chromatic response. This view was substantiated by the use of a blue micro-disk instead of a grey one.

These three pieces of evidence are summarized in the following plan:

Red receptor area	Blue receptor area	Red receptor area
Green receptor area	Green fixation point	Green receptor area
Red fixation point	Green receptor area	Blue fixation point

Each of these areas, so far as present evidence goes, contains roughly twenty-five photo-receptors. Each of the above areas contains predominantly receptors suggested by the name of the area, but, in addition, there are always present a few receptors responding to other coloured lights. This accounts for the general response of each area being somewhat diluted with white light. Thus when the red receptor area is stimulated the response is not red but pink.

The colour vision obtained by micro-stimulation of the human retina.

By H. HARTRIDGE. *Department of Physiology, St Bartholomew's Hospital, London*

Many years ago, Abney and Festing (1892) found, with their colour-mixture apparatus, that, as a spectrum is reduced in brightness, one part after another loses its colour. First yellow is replaced by white. At a somewhat lower intensity blue becomes black or dark grey. When the spectrum is examined at this stage, it is seen to contain two coloured areas, one in the red and orange, and the other in the green; between these there is a gradual change from red to white, and then from white to blue-green. Thus there is a form of dichromatic vision.

When extremely small colour filters are illuminated by skylight, and when these are viewed at such a distance that micro-stimulation of the retina takes place, again a form of dichromatism is found to be present. But while in a general way the dichromatism produced by micro-stimulation resembles that produced by reducing illumination, in detail they appear to differ. Thus a red micro-disk, besides producing various shades of pink, also produces at times shades of orange, salmon pink, flesh pink and buff which are quite different from the former pink. Similar tests with a blue-green micro-disk, besides producing various pale shades of that colour, also cause the observer to see shades of smoke grey, dove grey, turquoise, and cobalt blue which seem to be quite different from the original shades. Now there can be no doubt that the anti-chromatic response greatly modifies the colour of the blues and the yellows, as has been described previously. At the same time the colours reported above require an explanation. It is possible, in spite of their small size, that these micro-disks are stimulating more than one retinal receptor, so that a red, a green, and a blue receptor might be receiving stimulation in various proportions, so as to produce the shades of colour mentioned above. But another explanation is also possible, namely, that the tiny rays from the micro-disks are stimulating in turn modulators of the type discovered by Granit, by means of his micro-electrode technique, in the mammalian retina. It is hoped that further research will show whether this latter view is correct.

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Cluster formation by the foveal cones. By H. HARTRIDGE.

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In a previous communication I have referred to the fact that, in a random distribution, articles of a similar kind are frequently found grouped together in clusters. An example of cluster formation is the Lumière screen used for colour photography. There is evidence that in the human retina clusters of similar kinds of photo-receptors also occur. These clusters may be produced by chance, as they are in the Lumière screen, or by some factor which affects their distribution during foetal development. The evidence for the existence of clusters in the human fovea may be summarized as follows:

(1) Maximum acuity by monochromatic light is obtained over a small area of retina only. The average diameter of this area is from four to eight times

the diameter of a cone. Outside this area of maximum acuity is a medium acuity zone which has an outside diameter of from twelve to twenty-four times the diameter of a cone.

(2) Micro-stimulation of the retina has shown that neighbouring small retinal areas have different colour responses. Thus the fixation points for red light, green light, and blue light differ slightly in position. In this and other ways a provisional plan has been obtained of a small part of the fovea of the author's left eye. Some further details of this will be found in an accompanying communication.

The cluster hypothesis, outlined above, agrees with the evidence of visual acuity measurements when lights of different colour are used, eclipse blindness, the colour changes of an intermittent source of white light, and the retinal direction effect of Stiles and Crawford (1933).

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Synthesis of acetylcholine by non-nervous tissue. By R. S. Comline* (introduced by W. Feldberg). *Physiological Laboratory, Cambridge*

An enzyme system catalysing the formation of acetylcholine can be extracted by saline from acetone-dried brain and cholinergic nerves. Such extracts synthesize, aerobically and anaerobically, large amounts of acetylcholine in the presence of KCl, choline, NaF, eserine, cysteine and, in addition, adenosinetriphosphate (ATP), citrate and a substance called the 'activator', prepared by boiling saline extracts of acetone-dried brain (for references see Feldberg & Mann, 1946). Synthesis under similar conditions has now been studied in organs other than the nervous system, particularly in the human placenta and in the spleen of the horse, tissues which are known to contain large amounts of acetylcholine.

Human placenta.† In the presence of ATP, citrate and activator, 590–750 μg. acetylcholine are formed per g. acetone-dried placenta in 1 hr. at 37° C., 390–470 μg. without citrate, 80–160 μg. without activator, 60–65 μg. without ATP and 20–30 μg. without ATP, citrate and activator. The values of 590–750 μg. are higher than those obtained from brain preparations of dog and cat but lower than those from rat and guinea-pig. There is, however, this difference: with brain extracts the synthesis of acetylcholine is greatly enhanced by citrate; with placental extracts citrate has either no action or only a comparatively small effect; on the other hand the activator has a much

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† We wish to thank Prof. McCance and Dr Dean of the Department of Experimental Medicine for assistance in obtaining placentae.

more pronounced effect on placental extracts. Boiled placental extracts contain about 20% of the activator found in brain. As in corresponding experiments with brain extracts ATP can be replaced by phosphocreatine.

Spleen. In the presence of ATP, citrate and activator between 15 and 40 μg. acetylcholine are formed per g. acetone dried horse spleen in 1 hr. at 37° C. These values may not represent the true ability of the spleen to synthesize acetylcholine since these saline extracts contain a powerful inhibitor which reduces the synthesis by brain and placental preparations. For instance the synthesis of acetylcholine by brain extracts may be reduced as much as 90% in the presence of splenic extracts. This inhibitor is non-dialysable and is almost inactive after boiling; it is not present in acetone-dried blood and is not identical with methaemoglobin. Preparations from the spleen of dog and sheep, the fresh tissue of which contains only traces of acetylcholine, form 10–24 μg./g./hr. acetylcholine under optimal conditions. They also contain an inhibitor but in smaller amounts than in horse spleen.

Of other tissue extracts examined in the presence of ATP, citrate and activator, only heart auricle synthesized appreciable amounts of acetylcholine (up to 90 μg./g./hr.); the heart ventricle and the liver synthesized between 0–25 μg./g./hr.

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Smooth muscle of the inferior palpebra activated by adrenergic sympathetic nerves. By Z. M. Bacq and W. Isola. *Institute of Physiology, Montevideo, Uruguay*

In the inferior palpebra of the cat and dog there is a thick (0·5–0·7 mm.) layer of smooth muscle; its contraction, elicited by adrenalin or stimulation of the cervical sympathetic, lowers the lid, and its tonus contributes to the control of the opening of the palpebral fissure. In the superior lid of the cat and dog there is only a weak and irregular smooth muscle.

The innervation of the retractor of the inferior palpebra is anatomically sympathetic and physiologically adrenergic. All the ganglion cells are concentrated in the superior cervical ganglion.

The retractor of the inferior palpebra has a very steady tonus, it does not show any spontaneous movements; its responses to quantitative nerve stimulation are regular and suitable for accurate analysis. Cocaine has an outstanding potentiating effect; eserine has little or no action. Atropine decreases markedly the effects of nerve stimulation and of adrenaline. Adrenalytic and sympatholytic drugs (933 F., 883 F., yohimbine) abolish the response to adrenaline, but in many cases decidedly *increase* the contraction elicited by nerve stimulation. The acutely denervated retractor of the inferior palpebra is not very sensitive to intravenous adrenaline, and contracts when rather heavy doses of acetylcholine are given. Six days after aseptic removal of the superior cervical ganglion, hypersensitivity to adrenaline and to a lesser degree to acetylcholine is easily recorded.*

* A detailed paper is in preparation for the *Arch. int. Physiol.*

Phosphocreatine and the synthesis of acetylcholine. By W. Feldberg and Catherine Hebb.* *From the Physiological Laboratory, Cambridge*

The suggestion has been put forward that adenosinetriphosphate (ATP) and phosphocreatine which both act as phosphorylating substances in muscle may act in an analogous manner in the synthesis of acetylcholine by nervous tissue (v. Muralt, 1943; Nachmansohn, Cox, Coates & Machado, 1943). In the case of ATP, the effect on the anaerobic synthesis of acetylcholine in the homogenized brain tissue was first demonstrated by Nachmansohn & Machado (1943). The effect of ATP on both the aerobic and anaerobic formation of acetylcholine in saline extracts of acetone-dried brain powder was described first by Feldberg & Mann (1944–5). No similar experiments with phosphocreatine have as yet been reported. Recently Drs P. Eggleton and Nimmo Smith of Edinburgh University kindly supplied us with a sample of pure synthetic phosphocreatine (Ba-salt).

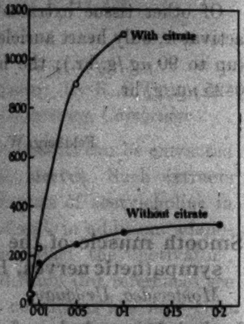

Fig. 1. Effect of phosphocreatine on the synthesis of acetylcholine in saline extracts of acetone-dried brain of the guinea-pig. Ordinates: μg. acetylcholine/g. acetone powder/hr. Abscissae: phosphocreatine-P in mg./ml.

Phosphocreatine was found to be able to replace ATP in the synthesis of acetylcholine in saline extracts of acetone-dried brain. In the presence of a suitable amount (0·05–0·1 mg. phosphocreatine-P/ml.) in the incubation mixture containing brain extract, eserine, choline, KCl, $MgCl_2$ and cysteine, acetylcholine is formed in large amounts exceeding sometimes the quantities synthesized in the presence of optimal concentrations of ATP.

Fig. 1 shows the effect of varying concentrations of phosphocreatine on the aerobic formation of acetylcholine; in the presence of citrate the synthesis is greatly enhanced. In this respect phosphocreatine behaves like ATP. Similarly, in the presence of phosphocreatine the synthesis of acetylcholine is slightly increased by *l*+glutamic and it is inhibited by pyruvic acid. It occurs both under aerobic and anaerobic conditions in the presence or absence of fluoride.

* Beit Memorial Fellow.

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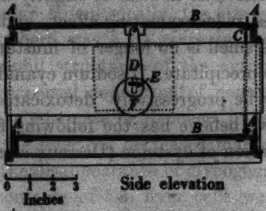
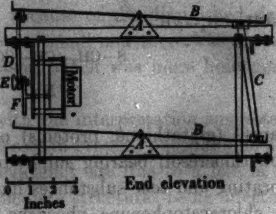
A simple rocking tray for staining histological preparations.

By R. A. M. Case. *R.N. Physiological Laboratory*

The apparatus consists essentially of a rigid metal frame constructed of light angled metal (e.g. aluminium) which is made to fit the tray supports inside an incubator. The top and bottom brackets carry bearings (*A*) through which spindles pass. Mounted on each spindle is a light but rigid metal tray (*B*) made of perforated aluminium. These trays are linked together by a pivoted strut (*C*).



Side elevation



End elevation

The apparatus is driven by a Smith 'Sec' electric clock motor, geared to revolve at 10–16 r.p.m. This is connected to the top tray by a pivoted arm (*D*) attached to a movable lock-nut (*E*) on the wheel of the drive (*F*). This enables the amplitude of swing to be varied.

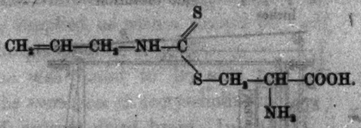
The apparatus possesses the following advantages: It may be left in the incubator with the trays horizontal, and thus serves as the normal shelves; it is silent in operation; it is self-starting; it may be run continuously for days without becoming hot, and it will carry a considerable load.

Simple demonstrations of the reaction of cysteine with chloropicrine and allylisosulphocyanide.

By Z. M. Bacq, V. Desreux, P. Fischer and Eugène Fredericq. *Departments of General Pathology and of Physical Chemistry, The University of Liège*

A 0·3% solution of cysteine-HCl of pH 7 in the presence of nitroprusside gives the purple colour characteristic of the thiol group. On addition drop by drop of chloropicrine (either a saturated solution in water or a 1% solution in ethyl alcohol), the colour fades and disappears; when the odour of chloropicrine is no longer present, a white crystalline precipitate appears (Bacq, Goffart & Angenot, 1940; Bacq & Desreux, 1942; Bacq & Fischer, 1943; Bacq, 1941; Bacq, 1942), which has been identified as pure cystine. In this particular case, the action of chloropicrine (CCl_3NO_2) is one of oxidation (Desreux, Fischer & Fredericq).

If one dissolves 16 mg. of cysteine-HCl in 20 c.c. half-saturated solution of allylisosulphocyanide buffered at neutral or slightly alkaline pH, after a few minutes the solution loses its lachrymating effect, and its stimulating action on the nasal mucosa; its smell is no longer of mustard but of rotten garlic (Bacq *et al.*). There is no precipitate. If sodium cyanide is added, a very large amount of vesicant may be progressively 'detoxicated' because the product of the reaction (which we believe has the following formula) is decomposed by cyanide with regeneration of cysteine (Desreux *et al.*):



$$CH_2{=}CH{-}CH_2{-}NH{-}C({=}S){-}S{-}CH_2{-}CH(NH_2){-}COOH.$$

Other natural substances (glutathione, proteins) or synthetic substances (mercaptans, thiophenol, thiocresol) bearing sulphydryl groups react easily with the vesicant, suffocating and tear substances like mustard gas (and its sulphone), chloracetone, chloracetophenone, phosgene, bromopicrine.

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Dietary protein and histological structure of liver tissue. By H. W. Kosterlitz. *Physiology Department, Marischal College, Aberdeen*

Water, protein, phospholipin, nucleic acid, glycogen and neutral lipids (including unsaponifiable matter) account for 96–97 % of the total weight of liver tissue (Table 1).

Table 1

Diet	Total weight	Water	Protein	Phospholipin	Nucleic acid	Glycogen	Neutral lipids
	μg./1000 liver cells						
60 % casein	8·20	5·72	1·46	0·250	0·106	0·244	0·137
20 % casein + 8 % yeast	7·07	4·99	1·17	0·212	0·092	0·229	0·131
18 % gelatin + 8 % yeast	6·85	4·77	1·04	0·194	0·090	0·218	0·291
8 % yeast	6·64	4·72	0·92	0·166	0·082	0·377	0·186
24 hr. fast after 20 % casein + 8 % yeast	4·99	3·50	0·92	0·166	0·076	0·006	0·147
24 hr. fast after 8 % yeast	4·71	3·37	0·80	0·150	0·074	0·008	0·120

The weight of the individual liver cell and its water content did not change significantly whether the rats were fed on diets adequate (20 % casein + 8 % yeast) or deficient (18 % gelatin + 8 % yeast or 8 % yeast) in protein. The loss in protein, phospholipin and nucleic acid was compensated by a gain in glycogen and neutral lipids. Histologically, the size of the liver cell showed no marked change, but there was a decrease in the chromophilic ground substance and the siderophil structures of the cytoplasm, which appeared rarefied because of the increased deposition of neutral lipids and glycogen. In rats fed on diets either adequate or deficient in protein and then fasted for 24 hr. the weight of the liver cell decreased markedly, due to the loss in water concomitant with the disappearance of glycogen. The protein, phospholipin and nucleic acid contents of the liver cells were decreased but their concentrations increased because of the diminished size of the cells. Histologically, the liver cells were small and there was an increase in the staining properties of the cytoplasm, which was more homogeneous than in the fed animals.

These findings agree with the interpretation suggested previously (Kosterlitz, 1944), viz. that the losses in protein, phospholipin and nucleic acid during fasting and in protein deficiency are due to a loss of liver cytoplasm, both particulate and interparticulate structures being affected.

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An apparatus for continuous injection of penicillin. By Major J. H. Bowie (introduced by E. J. King). *Indian Medical Service*

The disadvantages of prolonged penicillin administration with ordinary saline-giving sets or three-hourly injections in cases of late, infected, compound fractures of the femur among British troops evacuated to India from Burma, prompted the development of an apparatus for continuous, small volume injection.

The apparatus consists of a 2 ml. syringe fixed to the patient's thigh. A flexible needle takes the place of rubber tubing. The piston is driven by an electric motor through a speedometer cable. The syringe is steadily evacuated over a period of 24 hr. when it can be loaded automatically.

The chief advantages of the apparatus are: (1) constancy of injection; (2) reliability, supervision is unnecessary; (3) any number of patients in a ward can be treated simultaneously from one motor; (4) owing to the mechanical advantage inherent in rotary drive, the potential pressure in the syringe (approximately 52 lb./sq.in. eliminates the possibility of blockage in the injecting needle; (5) the drug is not in contact with substances such as rubber tubing which may have a deleterious action; (6) the patient is not confined to bed but to the length of his cable; (7) the extremely small volume in which the penicillin is injected reduces proportionately: (*a*) pain and local oedema, (*b*) deterioration of penicillin at room temperature.

The penicillin content of the serum was estimated by slide cell tests performed in large Petri dishes. The diluent used in the doubling dilutions was the individual's own serum obtained immediately before the exhibition of penicillin. Errors in the interpretation of the test, due to variation in the sensitivity of the test organism (N.C.T.C. No. 6571 A), were controlled by the determination of the sensitivity in serum in parallel with each day's tests. Further, a more exact figure for the final bacteriostatic dilution was obtained by the interpolation of a 2/3, 1/3, 1/6, etc. series between dilutions in the usual 1/1, 1/2, 1/4 series.

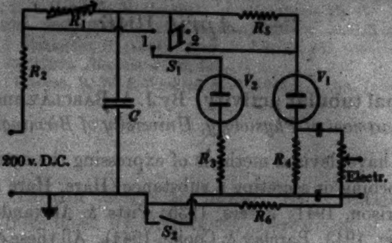
The serum levels in thirteen volunteers undergoing continuous injection of 28,000 to 120,000 units in 24 hr. were observed at 3 hr. intervals for 2 days. The blood level appeared to be remarkably constant during the last 43–45 hr., directly proportional to the dose and inversely proportional to the body weight.

The test results indicate that a dose of 25,000 units administered continuously over the 24 hr. will secure a constant blood level of 0·02 unit/ml. in a healthy man of 140 lb. weight.

An improved nerve-muscle preparation stimulator for student use.

By I. C. Whitfield (introduced by E. W. H. Cruickshank). *Department of Physiology, Marischal College, Aberdeen*

One of the drawbacks to the use of the 'neon-stimulator' which, for student 'nerve-muscle' experiments, is in general superior to the induction coil, has been the difficulty of obtaining two stimuli in quick succession by means of the rotating contact arms of the recording drum, as in summation experiments, etc. The difficulty is that the time of contact of each arm is comparable with the duration of the 'break' between them. Hence with a high charging resistance the condenser has insufficient time to recharge ready for the second stimulus while with a low one it may charge so rapidly that it discharges twice during each contact period giving four stimuli instead of two. In the circuit shown only one discharge can take place at each contact.



With S_1 in position 2 the circuit operates in the usual way, C discharging repeatedly through the neon lamp ('voltage stabilizer') V_1, at a frequency determined by the setting of R_1, as long as S_2 remains closed. With S_1 in position 1 the condenser discharges through both V_1 and a second similar lamp V_2, producing a stimulus across R_4 ($R_3 = 5 \times R_4$). V_2 has a slightly lower extinction potential than V_1 and continues to draw current after V_1 is extinguished. $R_2V_2R_3$ act as a potential divider which prevents C charging up to a high enough potential to 'strike' V_1 a second time as long as S_2 remains closed. As soon as S_2 is opened V_2 is extinguished and C charges up rapidly through the small resistance R_2. Thus in spite of the small value of R_2 only one pulse is produced across R_4 at each closure of S_2 irrespective of the relative times for which S_2 is opened or closed. R_5 is introduced to maintain the potential across R_4 the same in the two positions of S_1. R_6 is a high resistance allowing stray charges on the electrodes to leak to earth.

PROCEEDINGS

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3–4 *April* 1946

An index of renal tubular activity. By J. A. Barclay and R. A. Kenney.

Department of Physiology, University of Birmingham

Various workers have devised methods of expressing the activity of the renal tubules in reabsorbing or secreting a substance (Hare, Hare & Phillips, 1943; Harrison & Harrison, 1941; Peters, 1935; Pitts & Alexander, 1944; Smith, Ollayas & Winkler, 1943; Barclay & Cooke, 1944). All these expressions suffer from the disadvantage that their values cannot be compared from one animal or set of experimental conditions to another, since they all involve the use of the absolute amount reabsorbed. An index applicable to any subject or set of conditions requires certain osmotic relationships as the limits, since these always have the same significance regardless of other factors. A step in this direction was taken by Rehberg (1926), who compared the amount of substance reabsorbed with the amount that could be reabsorbed in a fluid isotonic with the glomerular filtrate. The value of this index can be found from the ratio $\frac{C'-C}{C'-1}$, where C is the concentration ratio of the substance being studied and C' that of the substance used to measure filtration rate (inulin, mannitol, creatinine).

With slight modification this ratio of Rehberg's can be made to serve as an index of activity applicable to a study of electrolyte reabsorption and secretion.

By comparing the difference between the amount of a substance excreted and that which would be excreted if only isotonic reabsorption occurred, with the amount reabsorbed isotonically, we arrive at the ratio $\frac{C-1}{C'-1}$. To this ratio we give the symbol E. The value of 1 signifies no elaboration subsequent to filtration, and if this condition applies the substance may be used to measure filtration rate. A value of zero indicates isotonic reabsorption ($C=1$). The range $0 \rightarrow 1$, where the substance is being reabsorbed along with water, we call 'passive reabsorption' ($C<C'$). A negative value shows that the reabsorption of the substance has outpaced that of water and so has become 'active'. A positive value greater than 1 indicates secretion ($C>C'$). We thus have a 'scale' calibrated between well-defined osmotic conditions.

Although water has been used as the 'yard-stick' for this index, it does not commit one to the view that all electrolytes are reabsorbed along with water. Proof of this has yet to be given.

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Heparin and trauma effects in blood. By S. Brandon Stoker and J. Pollard (introduced by D. Burns). *Department of Physiology, King's College, Newcastle-upon-Tyne*

Blood coagulation time (B.C.T.) as measured by Stoker's technique (Stoker, 1945) varies systematically after trauma (skin prick or tourniquet compression), the B.C.T. undergoing a preliminary shortening and then a prolonged lengthening. The increased coagulability is local or regional and varies directly with the extent of the trauma. The later increase in B.C.T. has a systematic variability similar to that shown by heparin (Stoker & Pollard, 1946) and may be obtained from blood taken from any part of the body, i.e. it is a general and not a purely local phenomenon. We have attempted to study separately the factors causing the local decrease in B.C.T. and the general increase in B.C.T., and have arbitrarily called the causative agents discharge A and discharge B.

Discharge A appears immediately after the infliction of the trauma and acts on B.C.T. like a chemically basic substance (Stoker & Pollard, 1946). It neutralizes the action of heparin on B.C.T. in vitro, and that of discharge B in vivo.

Discharge B acts on B.C.T. like heparin, neutralizing the action of toluidin blue, but not that of chlorazol fast pink, on shed blood. It is antagonistic to discharge A. It appears freely only when the skin is warm, suggesting that, with the amount of trauma used, the skin is its main source. By toluidin blue staining, the presence of heparin granules may be demonstrated in the skin (Jorpes, 1939).

The amount of discharge B has been estimated by methods based (*a*) on the action of toluidin blue, and (*b*) on the amount of anti-heparin bodies present in the blood. After a skin prick, the maximum discharge is equal in effect to 24–36 Toronto units of heparin per 100 ml. of blood. The release of discharge B begins when trauma occurs, but owing to the local release of discharge A and the general release of discharge B the lengthening of B.C.T. is delayed at points near the site of the trauma. It is produced generally over the body as a result of nervous activity, not continuously, but in a series of flows, and does not seem to vary in amount in proportion to the degree of trauma.

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The life-span of the erythrocyte. By J. N. Mills.

Fellow of Jesus College, Cambridge

If $F(t)$ is the fraction of a population of simultaneously released erythrocytes which survives after time t, and $G(t)$ the disappearance function for a random isolated sample of blood including cells of all ages, it can be shown that $F(t)=G'(t)/G'(0)$. Functions of the form $G(t)$ are obtained when the disappearance of erythrocytes transfused into a recipient with antigenically distinct cells is studied. Callender, Powell & Witts (1945) derive a similar equation which obscures the dependence of all values of $F(t)$ upon the initial slope of the disappearance curve.

It can also be shown that the mean life span of the erythrocyte is $-G(0)/G'(0)$, or the intercept on the time axis made by a tangent to the disappearance curve at time zero. Callender *et al.*, in estimating a mean life span of somewhat over 100 days, make the unproved assumption that a linear disappearance observed for some weeks can be extrapolated to zero time. If this premise is incorrect the method may grossly overestimate the mean life span. A brief initial period of rapid fall of $F(t)$ would probably be overlooked or ascribed, perhaps, to a slight haemolytic reaction; but if due to the natural viability of the erythrocyte a loss of 4 % of transfused cells in the first 2 days would imply a mean life span of 50 days, however the disappearance curve subsequently behaved. If this continued as a straight line reaching zero at 100 days the implication would be that 51 % of the newly formed erythrocytes only survived for 2 days whilst the other 49 % survived for 100 days. This supposition may appear intrinsically improbable, but it seems the easiest way of reconciling the results of the transfusion method with the figure of 42 days for the mean life span of the erythrocyte obtained by Baar & Lloyd (1943) by the reticulocyte method.

Here two basic assumptions are made. The initial linear disappearance of reticulocytes in incubated blood is extrapolated to the time axis to determine the mean maturation time; the mathematical treatment indicated above shows that this procedure is correct, whether or no the later slower fall is due to unphysiological conditions. It is also assumed that all erythrocytes enter the circulation as reticulocytes; but if this be not correct then the method overestimates the mean life span, and the discrepancy from the transfusion method becomes worse. The present weakness of this method is that the limits of statistical error of the estimates have not been defined and may be very large.

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Diuretic action of cyanate. By F. Schütz. *Department of Pharmacology, University of Birmingham*

In addition to its previously described actions (Schütz, 1945), cyanate was found to be a potent diuretic. Small, non-toxic doses, given intramuscularly to rats, are followed by a marked diuresis, which, in contrast to other diuretics, occurs even if no excess water is given. The experiments summarized in Fig. 1 were made on albino rats (100–150 g.) without any excess water being given previously. It can be seen that cyanate was more potent than a number of other well-known diuretics. The ineffectiveness of ammonia shows that the potency of cyanate is due to the CNO group, and not to the ammonia, into which injected cyanate may break down in the body. Subcutaneous injections of cyanate were found to be less effective than intramuscular.

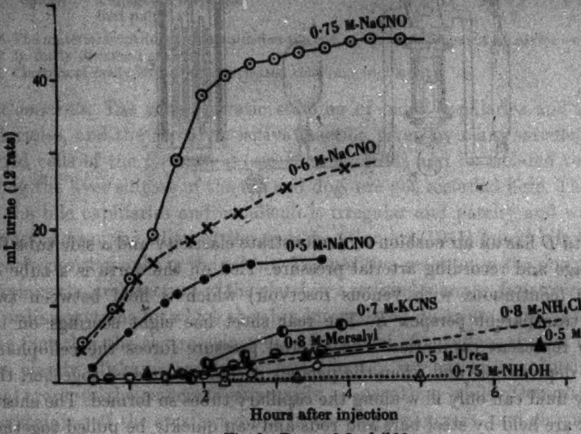

Fig. 1. Doses: 0·3 ml./100 g.

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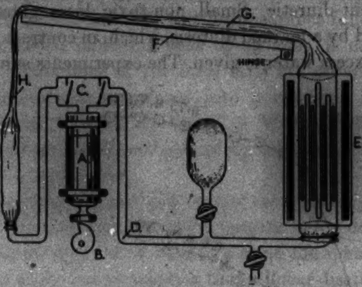
Mechanical model of heart and circulation. By H. P. Gilding and K. A. Webb. *Department of Physiology, University of Birmingham*

The figure shows the essential details of this model. The pump *A* is an all-glass 20 c.c. syringe with detachable nozzle. The nozzle is replaced by a rubber bung through which passes one of the three nozzles of the valve chamber *C*. The rate of pumping is controlled by a variable resistance which regulates the speed of the motor driving the cam *B*. The stroke volume is dependent on the height of the column of fluid in the cellophane vena cava *H*. This column can be altered at will by lowering the platform *F* and allowing more fluid to flow from the cellophane venous reservoir *G* into the vena cava, or, alternatively, by pressing on the reservoir thus simulating muscle action.



The aorta *D* has an air cushion to demonstrate elasticity and a side tube for haemorrhage and recording arterial pressure. Tied on the aorta is a tube of cellophane (continuous with venous reservoir) which is held between two sheets of transparent perspex *E*. The rear sheet has eight scorings on its surface to represent capillaries. The arterial pressure forces the cellophane into these depressions, and when the perspex sheets are pressed together, the circulatory fluid can only flow along the capillary tubes so formed. The sheets of perspex are held by steel bars and rods and can quickly be pulled together or apart by operating a cam situated behind the apparatus. By this means the peripheral resistance can be altered at will. The perspex is illuminated from behind so that it is possible, with a coloured fluid circulating, to see (*a*) constriction with only capillaries apparent, or (*b*) the whole cellophane tube (indicating dilatation).

The colouring matter must not stain the cellophane, otherwise the capillaries would be invisible. Carmine is a suitable dyestuff.

The pump rate can be varied from 20 to 80 beats/min., and blood pressure from 30 to 300 mm. Hg, and outputs from nil up to 500 c.c./min.

Differences in localization, by histochemical means, of alkaline phosphatase within the same organ of different experimental animals. By F. Jacoby. *Department of Physiology, The Medical School, Birmingham*

In the course of histochemical investigations (Gomori's method) on the distribution of alkaline phosphatase in various tissues of different animals certain species differences were encountered with a degree of regularity which allows them to be summarized as follows:

Table 1

		Rat	Guinea-pig	Dog*
Liver:	Bile capillaries	+	−	+
	Epithelium of bile ducts	−	−	+
	Subepith. C.T. of ducts	(+)	−	−
Pancreas:	Centro-acinar cells and intercalated ducts	−	−	+ +
	Epithelium of larger ducts	−	−	+
	Subepith. C.T. of ducts	(+)	−	−
	Islets	−	−	+†
Spleen:	Malpighian corpuscle	−	−(+)	+ (periphery)
	Red pulp	+‡	+‡	−

* The material from dogs was obtained at the end of a renal experiment involving a saline drip.

† In finely dispersed granules.

‡ Clusters of fairly large mono-nucleated cells (macrophages?).

Comments. The rather erratic staining of blood capillaries and occasional arterioles, and the heavily positive reaction given by many circulating white blood cells of the guinea-pig (see Bourne, 1944) and by isolated cells in and along the liver sinuses of the rat and dog, are not recorded here. The staining of the bile capillaries and canaliculi is irregular and patchy and was mainly observed near the edges of the section. Gomori (1941) found bile capillaries clearly positive only in the rabbit. The positive reaction in the dog's pancreas displays the architecture of the gland in a striking manner, far superior to that of ordinary stains by which the thin intercalated ducts are difficult to discern. The fact that the centro-acinar cells form part of this duct system is brought out clearly. Takamatsu (1939), investigating various species (including the dog), reports the pancreas summarily as negative, whilst Gomori mentions the staining of the smallest ducts in the dog's pancreas, and claims that the epithelium of the larger ducts is more or less positive in practically all species (including the rat and guinea-pig). He makes the same statement concerning the epithelium of the intrahepatic bile ducts. Neither of these latter statements are borne out by the present study. The question whether such discrepancies are due to different functional states must be left for future work.

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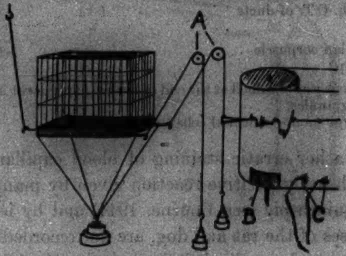
A simple device for recording the action of drugs on motility of rats

By F. Schütz. *Department of Pharmacology, University of Birmingham*

Rats exhibit regular sleep habits; they sleep in bunches during most of the day and are awake for many hours after sunset. If not asleep they usually move about, so that a record of their movements enables us to assess the action of drugs on sleep. No new principles are involved in the simple device shown below. The cage, containing 4–6 rats, is suspended on three points. Two wires are led over ball-bearing pulleys (*A*). Two such cages, divided by a screen, are connected as shown in the figure with a slow-moving kymograph, giving a tracing of 8 ft. length in approximately 6 hr. *B* and *C* are a conducting surface and wire brushes respectively, arranged to stop the motor automatically after one revolution of the paper. The conducting surface (*B*) is provided by either a strip of tinfoil or a single application of colloidal graphite.

The method records well the action of small sleep-preventing doses of amphetamine, ephedrine, etc., when given during the normal sleep period of the rat (daytime). Sleep induction by drugs, and the length of such sleep, can likewise be tested during the period of normal wakefulness (night). The method also proved useful in recording the action on sleep of some natural substances (for hydration through posterior pituitary extracts and water see Schütz (1944), and Millington & Schütz (1946), and of cyanate see Schütz (1945)), which cannot be tested like common hypnotics because they do not produce deep anaesthesia. The establishment of minimum effective doses may be made by this method. These doses are of special interest for this group of substances since they enable comparison of drugs given in non-lethal doses to be made. The use of the LD50 compares the toxicity in a range of doses far higher than the equivalent in practical use.



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PROCEEDINGS
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An experimental analysis of the human jugular pulse tracing.

By Ian F. S. Mackay. *Department of Physiology, The University, Sheffield*

The logical approach for an experimental analysis of the venous pulse tracing would be to eliminate alternatively the venous and arterial components and then, by examination of the resultant tracings, to determine which of the two factors is responsible for the individual waves.

Venous 'occlusion'

Fig. I.

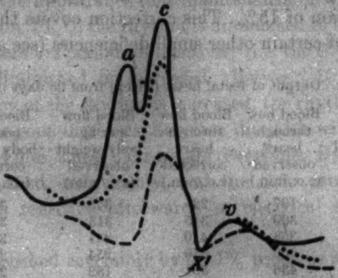

Fig. 2. Comparison of venous pulse waves before and after 'occlusion'. —— Before 'occlusion'. ···· During 'occlusion'. --- After 'occlusion'.

With the aid of a tilting table and tourniquets (details of method will be published later), 'occlusion' of the venous and arterial pulsations can be achieved. 'Occlusion' of the arterial factor can be obtained by the use of chest tourniquets, but, as might be expected, this procedure also 'occludes' the venous pulsation. Venous 'occlusion' produces a temporary reduction in the venous return and a disappearance—partial or complete—of certain waves in the phlebogram. In the tracing in Fig. 1 it can be seen that after the 'occlusion' the '*a*' wave has almost completely disappeared, the '*c*' wave is reduced and the '*v*' wave is unaltered. Control tracings showed no change.

To demonstrate these changes more clearly copies of three pulsations from another tracing (i) before, (ii) during and (iii) after venous 'occlusion' were super-imposed (see Fig. 2).

These results would suggest that the '*a*' wave is of venous origin. The '*c*' wave is made up from venous and arterial elements and, finally, that pulsations from extravenous sources enter into the formation of the '*v*' wave.

The output of the heart of the foetal sheep. By JOSEPH BARCROFT (*Unit of Animal Physiology, Agricultural Research Council*), and D. S. TORRENS (*Physiological Laboratory, Trinity College, Dublin*)

The output of the heart has been measured by a cardiometer luted with a special lute made of beeswax, paraffin and lanoline, which, when warm, adheres to the tissues. The following observations have been obtained from the 95th day onwards (gestation period 147 days). Because all possible sources of error are such as to diminish the blood flow through the heart, the maximum reading has been taken, though the readings in a single heart have been reasonably concordant. In Table 1 the observed figures are given, together with those obtained by adding a correction of 15%. This correction covers the circulation in the coronary system and certain other small deficiencies (see *J. Physiol.* **82**, 501).

TABLE 1. Output of foetal heart (sheep) from 95 days onwards

Serial no.	Weight of foetus (kg.)	Foetal age (days)	Blood flow through heart observed (c.c./min.)	Blood flow through heart corrected (c.c./min.)	Blood flow per kg. body weight observed (c.c./min.)	Blood flow per kg. body weight corrected (c.c./min.)	Anaesthetic
685	0·57	95	197	225	345	395	Chloral
683	0·96	106	300	345	313	360	Chloral
686	1·05	108	358	410	341	390	Chloral
682	2·2	120	560	640	254	295	Chloral
708	2·4	123	436	501	182	210	Chloral plus spinal
689	1·64	129	334	380	204	235	Chloral
649	2·81	136	690	800	245	285	Spinal
709	4·1	141	676	770	165	190	Chloral plus spinal

The maximum observed output was 800 c.c./min.
The output per kg. of foetus on the whole falls as gestation proceeds.

Effects of temperature and anaesthetics on spontaneous rhythmic contractions of body-wall muscle of the earthworm. By W. F. Floyd. *Department of Physiology, Middlesex Hospital Medical School, London*

Strips of body-wall muscle of the earthworm were suspended in frog Ringer's solution in a leech bath and connected with a very light gimbal lever. Both longitudinal and circular strips were used. The former were usually cut from the dorsal or lateral parts of the body wall: the latter were prepared as follows. Two transections were made, two or three segments apart, and the 'ring' of earthworm body so isolated was cut dorsally in the mid-line and the gut and intersegmental septa removed: nerve cord, ganglia and nerves were left intact. The segments used were mostly from the region immediately anterior to the clitellum.

Both longitudinal and circular muscle strips show spontaneous rhythmic contractions, of period 10–60 sec. at ordinary room temperatures (12–15° C.), as was first shown by Straub (1900), but his circular muscle rhythm was of much greater frequency and of small amplitude, and clearly different from the present results. Straub's observations on longitudinal muscle strips were confirmed by Wu (1939). Trendelenburg (1916) found only occasional spontaneous rhythmicity of the circular muscle in preparations with nerve cord removed, and the contractions were small.

The spontaneous rhythm of the circular muscle strips in the present experiments was usually well marked; the extent of the contractions represented a 5–50% shortening with a force of the order of 100 mg. wt. The optimum temperature favouring the appearance and maintenance of the rhythm is in the range 10–15° C. Below 10° C. the rhythm is diminished in frequency and often in amplitude, and there are periods of inactivity (i.e. the rhythm is broken up); the tone may be diminished or increased. Between 15 and about 30° C., initially the frequency of the rhythm is increased, the rhythm tends to become more regular and the tone increases, but may diminish with disappearance of the rhythm, after 2–4 min. These changes are reversible. Between 30 and 40° C. the rhythm is abolished, irreversibly, and the tone usually increases. The preparation remains sensitive afterwards to electrical stimulation and to acetylcholine.

The rhythm is abolished reversibly by 0·02% procaine in Ringer's solution: with higher concentrations the effect is not always reversible. Body wall is thus more sensitive than earthworm gut, for which Millot (1943) used 5% procaine to abolish the rhythm. 1% ethyl alcohol greatly diminishes the rhythm and 10% (the strength used in other experiments for anaesthetizing the earthworm) abolishes it, reversibly.

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Continuous measurement of oxygen concentration in physiological media. By D. K. Hill. *Department of Physiology, University of Cambridge*

The oxygen concentration in saline or other physiological fluids may be recorded continuously, and rapid changes may be followed by means of a platinum electrode. The principle, fully described by Davies & Brink (1942), is briefly as follows. The electrode is maintained at a negative potential of about 0·8 V. relative to the bulk of the fluid, and molecular oxygen is reduced at the surface of the platinum according to the following reaction:

$$O_2 + 2\text{H-ions} + 2\text{ electrons} = H_2O_2.$$

The current which passes through the electrode is limited by the rate at which oxygen can diffuse to the surface of the platinum and is proportional to the oxygen concentration in the medium.

In physiological fluids no other reaction takes place at this voltage and the current is determined by the oxygen alone.

Bronk (1944), Bronk, Brink & Davies (1941) and Davies & Brink (1941) have utilized the method for following the respiration of brain and nerve.

The fluctuations in the current which may be caused by movements of the fluid in the region of the electrode surface are minimized by reducing, as far as possible, the area of exposed platinum (platinum wire down to 1 μ diameter may be used, although for most purposes the wire need not be thinner than about 10 μ), and by recessing the active area of platinum to a depth of 50–100 μ in a glass sheath. The speed of response is reduced by this recessing, and changes in oxygen concentration occurring in less than about 5 sec. are not followed with accuracy. Electrodes of this type pass about 10^{-9} to 10^{-11} amp. A further reason for reducing the size of the electrode surface is to avoid having to make correction for the oxygen used by the electrode itself: this complication might arise when measuring the oxygen tension in very small quantities of fluid.

Various forms of the electrode may be used for different purposes, such as for continuously recording the oxygen consumption of a strip of plain muscle from the snail (*Helix aspersa*).

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PROCEEDINGS
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The oxygen consumption of the sheep foetus. By Joseph Barcroft and S. R. Elsden. *Cambridge Unit of Animal Physiology, Agricultural Research Council*

Carlyle (1945) communicated an ‘interpretation of the total oxygen consumption of the sheep foetus from estimations of the Q_{O_2}’s of individual tissues’. The results from 112 days onwards agreed very closely with those given by other observers for the intact and living foetus. The estimates of Barcroft & Torrens (1946) give the opportunity of recalculating the oxygen used by the foetus by combining measurement of the oxygen difference between the arterial and venous bloods with the measurements of cardiac output made by Barcroft & Torrens. The assumption is made that the flow through the placenta is half that through the heart.

Oxygen used by sheep foetus

Sheep	Weight (kg.)	Foetal age (days)	Blood flow through placenta c.c./min.	Blood flow through placenta c.c./kg./min.	ΔO_2 as between umb. art. and vein (c.c./100 c.c.)	Oxygen used by foetus c.c./min.	Oxygen used by foetus c.c./kg./min.
685	0·57	95	113	198	6·3	7·1	12·5
683	0·96	106	173	180	5·3	9·2	9·6
686	1·05	108	205	185	4·05	8·3	7·9
708	2·04	123	251	105	3·7	9·3	3·9
649	2·81	136	400	143	3·6	14·4	6·6
709	4·10	141	385	95	5·7	22·0	5·4

The figures for the O_2/kg./min. are, with one exception, substantially higher than those given by Carlyle’s computation from tissue slices.

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Sympathin in adrenergic nerve fibres. By U. S. v. Euler.

Department of Physiology, Karolinska Institutet, Stockholm

Extracts of various mammalian organs contain a sympathomimetic substance having a catechol nucleus, but differing in certain respects from adrenaline (Euler, 1946). Extracts of the thoracic sympathetic chain and the nerves of the splenic artery of cattle and horses were found, when tested on the blood pressure of the cat, to contain large amounts, up to 100μg. adrenaline equivalent, per g. tissue. After purification, the extracts showed actions which consistently paralleled those of *nor*adrenaline, when tested on the blood pressure of the cat, the uterus and intestine of the cat and the rabbit, the pupil and the heart of the cat. Whenever the action of *nor*adrenaline differed from that of adrenaline the same was true for the active extracts. Similarly, the action of the extracts after large doses of ergotamine and dihydro-ergotamine (Sandoz), as in the fluorescence test of Gaddum & Schild, equalled that of *nor*adrenaline and differed from adrenaline in the known characteristic ways.

The biological action of the adrenergic nerve extracts, closely imitating those of *nor*adrenaline, revealed the typical preponderance of motor actions over inhibitory actions when compared with adrenaline. The active substance accordingly is related to the postulated sympathin E of Cannon & Rosenblueth. On the other hand, the active substance, like *nor*adrenaline exerts undisputable inhibitory actions (cf. Euler, 1946, Fig. 1), which seems to make the assumption of a special sympathin I superfluous.

Since the active substance can be directly extracted by water or saline from fresh nerve preparations, it is probably identical with the mediator of the adrenergic nerve fibres. The mediator would thus be related to, but clearly different from, adrenaline, which seems to be present only in the adrenals, and in scattered groups of chromaffine cells. The active substance is present in, and probably liberated physiologically from, the whole length of the adrenergic nerve fibre and not only in the 'nerve endings'. The active substance also occurs in considerable amounts in sensory nerves to the skin and, to a lesser degree, in spinal roots. The grey sympathetic rami of the horse were found to contain a far larger amount of the substance than the white ones.

It is suggested that the adrenergic neuro-humoral principle, presumably identical with *nor*adrenaline, should be termed 'sympathin' in order to avoid the introduction of new names, and in recognition of the original work of Cannon & Bacq (1931), notwithstanding the fact that the term has been used with varying meanings.

A full account of the experiments, here briefly referred to, will appear in the *Acta Physiologica Scandinavica*, 1946.

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A histamine dilatation abolished by atropine.

By J. A. C. Knox. *King's College, London*

In 1936 McDowall & McWhan showed that when the hindlimbs of a cat were perfused with a calcium-free saline solution of pH 8 the dilator effect of small doses of histamine was greatly enhanced. It has now been found that the dilatation produced by histamine under such circumstances is abolished or greatly diminished after atropine has been injected into the perfusion fluid. Occasionally, a dilator dose of histamine will, after atropine has been given, cause a definite constriction.

A similar histamine dilatation abolished by atropine has been produced, but not constantly, in the purely arterial mesenteric preparation described by Dale & Richards (1918).

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Recent work on the lipotropic factors.

By C. H. Best, F. Hoffmann, C. C. Lucas and J. Talesnik. *Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada and Institute of Physiology, University of Chile, Santiago, Chile*

A reinvestigation of the relative effectiveness of the lipotropic agents—choline and inositol—in preventing damage to the liver and kidneys of young rats, has been made. Inositol is always less active than choline in limiting deposition of lipids in the liver and under certain circumstances is completely ineffective. The kidney lesions are not favourably affected by inositol. In rats with haemorrhagic kidneys, i.e. exhibiting the effects of a profound choline deficiency, a delay in the disappearance of the response of the voluntary muscles to stimulation of the cut sciatic nerve has been consistently demonstrated, but the mechanism by which lack of choline produces this change has not as yet been elucidated. Our present working hypothesis is that the interference with the metabolism of the phospholipids in nerve may play an important part in this phenomenon.

Records and findings obtained during studies of the renal circulation in the rabbit with special reference to vascular short-circuiting and functional cortical ischaemia.

By A. E. Barclay, P. Daniel, K. J. Franklin, M. M. L. Prichard and J. Trueta. *Nuffield Institute for Medical Research, Oxford*

The clinical picture in post-traumatic kidney failure ('traumatic uraemia') is predominantly one of uraemia and anuria, and the histological picture one of cortical damage. For reasons outlined by one of us (Trueta, 1945), we have been investigating the possibility that the mechanism concerned is a reflex one, and in the course of our research we have secured evidence (*a*) that vascular short-circuiting can occur in the kidney, and (*b*) that the blood supply to the cortex in particular can be reduced, by such short-circuiting, to any degree up to complete ischaemia.

The evidence, obtained in a series of rabbits subjected to various experimental procedures, includes the following:

(1) Radiological studies showed that in certain animals the normal circuit time from renal artery to renal vein was reduced by one-third or one-half. In one animal this reduction was concomitant with marked decrease in calibre of the renal artery.

(2) In a number of animals, red streamlines were seen in the general blue stream of the blood in the renal vein. In a further number of animals, the whole of the renal vein blood, as viewed from the ventral aspect, became arterial in colour. In one animal, pulsation of arterial type was visible in the renal vein with the appearance of the red stream.

(3) In a number of animals, dyes injected into the arterial side of the circulation appeared in the renal vein but did not stain the renal cortex. On section, however, intense staining of the medulla was apparent.

(4) In a number of animals, red streamlines appeared in the renal vein after the cortex had paled and while it still remained pallid.

Evidence (1) and (2) above shows that short-circuiting of various degree can occur in the kidney and, (3) and (4) above, that it can be accompanied by functional cortical ischaemia. This suggests a much greater degree of short-circuiting than could be accounted for by anatomical evidence so far published, and our own investigations, though they are not yet complete, incline us to believe that the by-passing is effected, at least in part, via the vasa recta.

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Radio-micrography: a preliminary communication. By A. E. Barclay, P. Daniel, H. M. Powell and M. M. L. Prichard.

Nuffield Institute for Medical Research, Oxford

Certain observations made during circulatory studies in rabbits called for a closer investigation of the blood flow through the kidney, and, in particular, for a detailed study of the renal vascular system. Good visualization of the intra-renal vascular distribution, including that of interlobular arteries and veins, was obtained by means of stereoscopic radiographs of the kidneys (both whole and in section) after injection of various radio-opaque substances. Ordinary radiographic techniques, however, gave insufficient definition for a study of the more minute structures, i.e. those not visible to the naked eye.

Experiments have accordingly been started with a view to developing a technique of radio-micrography. First results have been obtained with the use of an old gas X-ray tube of the Shearer type, ordinarily employed in the determination of crystal structure. This tube, which emits a high proportion of soft radiations through its thin aluminium foil window, has a copper target and a very small focal spot. High resolution film has been used and early results have permitted photographic enlargement of at least 28 diameters. So far exposures have been largely confined to sections of kidneys of about 50 to 250μ, taken from rabbits which had been intravenously injected during life with radio-opaque media, but sections of similar and lesser thicknesses are being prepared from a number of organs for radio-micrographic study.

It seems likely that this new technique, which at present involves the injection of radio-opaque media, will do much to bridge the gap which has hitherto existed between macroscopic and microscopic methods of studying biological structures. A possible extension is the use of radio-micrography, without injection of radio-opaque media, for the detailed study of tissues. This would involve more accurate control and selection of the type of radiations used, but the advantages which would accrue from such a technique are so great that its possibilities are being explored.

PROCEEDINGS

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Certain physiological variations in the haemoglobin levels of sheep.

By W. Thomson, A. M. Thomson and D. P. Cuthbertson, *Rowett Research Institute, Aberdeen*

Haemoglobin was estimated on ear-prick blood with a standardized Haldane-Gowers apparatus. The following experiments were carried out to elucidate the nature of individual fluctuations.

(1) Fifteen ewes were caught and held quietly for 30 min., frequent blood samples being taken during this period. The haemoglobin fell by about 10% in the first 10 min., unaccompanied by a fall in the respiration rate.

(2) 354 double estimations were made over a period of 4 months on seventy-seven ewes, on capture and after holding quietly for 15 min. In 325 pairs (91·8%) there was a decline to about 90% of the initial level; in seventeen pairs (4·8%) there was no change; and in twelve pairs (3·4%) there was an increase averaging 6%. The largest change recorded is a fall of 18% on the Haldane scale.

(3) The haemoglobin level rose about 10% when resting (held) sheep were confronted by a barking dog or were chased for 2 min. Thereafter a fall to the resting level took place. Similar changes were observed in jugular blood taken simultaneously from a few animals. No parallel changes in serum calcium took place.

(4) 1–1·5 ml. of 1 : 1000 adrenalin injected subcutaneously into two sheep caused an immediate increase in the capillary blood-sugar level and a rise in the respiration and pulse rates. There was, however, no effect on the haemoglobin level.

(5) Immediately after parturition and during the lactation period the haemoglobin level tended to remain steady or to rise when ewes were caught and held in the usual way. This may be associated with sustained or mounting excitement on separation from the lambs.

(6) Fourteen ewes due for operation were not given the usual 6.30 a.m. meal. The haemoglobin level was estimated at 9 a.m. after capturing and holding for 15 min., and again later when the animal was anaesthetized. The average readings were: on capture, 77%; after holding, 75%; under chloral hydrate

anaesthesia, 67 %. The absence of a significant fall during the holding period may be associated with excitement due to missing the customary morning meal. A trial on one of these ewes produced a normal fall on holding at 9 a.m. on the day before operation, after the usual 6.30 a.m. meal.

The absence of an effect with adrenalin increases our doubt as to whether fluctuations in size of the small spleen of the sheep could give effects commensurate with those observed. A decrease in plasma volume shown to occur in man after exercise (Kaltreider & Meneely, 1940) seems more likely.

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The effect of extracts of gastric and intestinal mucosa on the secretion of HCl by the cat's stomach. By A. A. Harper, *Department of Physiology, Manchester University*

Since Edkins (1906) published his observations on gastrin, many workers, notably Ivy and his colleagues (1932), have claimed that the gastric hormone, if it exists, is histamine. More recently, Komarov (1942) has extracted a histamine-free protein-like material from the pyloric mucous membrane, which on injection stimulated secretion of acid by the stomach.

The question has been reinvestigated by making extracts of hog gastric and intestinal mucosa by a modification of Mellanby's method for the preparation of secretin (Mellanby, 1932). The mucosa is extracted with 4 volumes of acid alcohol or of 60 % alcohol. The alcohol is distilled off and the active material precipitated either by saturation of the concentrate with NaCl, or by the addition of bile salts, followed by extraction of the bile salt precipitate with alcohol.

Extracts of the pyloric mucous membrane on intravenous injection stimulate the secretion of HCl by the cat's stomach. Extracts of the mucosa of the upper part of the small intestine also have some stimulant action upon acid secretion by the stomach, and in addition contain secretin and pancreozymin. Extracts of the mucosa of the body of the stomach and of the lower part of the small intestine are inactive. The active material in these extracts does not dialyse through a cellophane membrane, and is destroyed by digestion with pepsin. The extracts have no depressor action on the blood pressure of the cat.

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The effects of fasting on the nucleic acid content of rat tissues. By J. N. DAVIDSON, *Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1

When weanling rats are fasted for 48 hr. and then fed stock diet *ad libitum* for a further 48 hr., the body weight falls and rises again almost to the initial value, the liver weight falls and then rises above the initial value, the weight of the spleen falls and rises to the initial level, while the weight of the thymus falls to a very low level on fasting, and rises only slightly, if at all, on feeding. These observations are in agreement with those of Andreasen (1943).

The principle employed by Schmidt & Tannhauser (1945) has been used in estimating the nucleic acid content of the liver, spleen and thymus. After removal of acid-soluble and lipid phosphorus, the extracted tissue is incubated with warm alkali. The phosphorus which remains acid-insoluble after this treatment is referred to here as the 'deoxyribonucleic acid fraction', while the phosphorus which becomes acid-soluble is referred to as the 'ribonucleic acid fraction'. On fasting, the 'ribonucleic acid fraction' decreases in the liver, in the spleen and, most of all, in the thymus. On feeding, this fraction is restored to the original level or above it in the liver, is partially restored in the spleen, and is restored only to a slight extent, if at all, in the thymus.

During fasting, the 'deoxyribonucleic acid fraction' falls to a very low level in the thymus, but does not fall in the liver (cf. Davidson & Waymouth, 1944; Davidson, 1945). On feeding, this fraction may increase slightly in the liver, but remains at a low level in the thymus.

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Effect of adenosinetriphosphate and creatine phosphate on the perfused superior cervical ganglion of the cat. By W. FELDBERG* and CATHERINE HEBB†, *Physiological Laboratory, Cambridge*

Phosphorylating substances such as adenosinetriphosphate (ATP) and creatine phosphate (CrP) are involved in the synthesis of acetylcholine (Nachmansohn & Machado, 1943; Nachmansohn & John, 1945; Feldberg & Mann, 1945; Feldberg & Hebb, 1946; Torda & Wolff, 1946). ATP also causes contraction of normal and denervated muscle (Buchthal, Deutsch & Knappeis, 1944; Buchthal & Folkow, 1944; Buchthal & Kahlson, 1944, 1946). Experiments were made on chloralosed cats to find any effect of these substances on sympathetic ganglion cells, using the nictitating membrane as an index of stimulation, with the following results:

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* With a grant from the Medical Research Council. † Beit Memorial Research Fellow

(1) The arterial injection into the ganglion of the Na salt of either ATP or CrP stimulates the ganglion cells in relatively large doses only. Expressed in terms of the labile P the threshold dose is about 0·1 mg.; this corresponds to approximately 1·6 mg. free ATP and 0·7 mg. free CrP.*

(2) The effects are not dependent on the acetylcholine metabolism of the preganglionic endings, since they are obtained 8–9 days after cutting the cervical sympathetic, when the ganglion is practically free from acetylcholine and its ability to synthesize it is known to have been lost.

(3) It is unlikely that the effect of ATP is due to the splitting off of its labile phosphate grouping, since muscle as well as yeast adenylic acid (adenosine-monophosphate) also stimulates the ganglion. The stimulating actions of ATP and CrP, as well as adenylic acid, are, however, related to their phosphorus since (1) creatine and adenosine which contain no phosphate had no action on the ganglion, and (2) effects similar to that of ATP and CrP could be produced by the injection of sodium pyrophosphate or sodium triphosphate. In fact, the effects of these substances were greater for an equivalent P content than those of either ATP or CrP.

We should like to thank Dr P. Eggleton and Dr R. H. N. Smith for preparing a sample of synthetic barium creatine phosphate.

* Kahlson and Lundberg have found independently a similar action of ATP on the sympathetic ganglion, while Emmelin and Kahlson have observed that ATP also causes an output of adrenaline from the suprarenals. These experiments are as yet unpublished, but Prof. Kahlson (Lund) has informed us of the results and given us permission to refer to them in the present communication.

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The influence of sex on liver cytoplasm. By Rosa M. Campbell and H. W. Kosterlitz, *Physiology Department, Marischal College, Aberdeen*

In both male and female rats the amount of cytoplasmic material (non-glycogen non-lipid solids) present in the liver is directly proportional to the logarithm of the casein intake. The regression coefficients show that the male rat forms very significantly greater quantities of 'labile' liver cytoplasm than does the female rat (Fig. 1). When rats are transferred from the stock diet to a protein-free diet, the loss in liver cytoplasm can be expressed by a curve containing an exponential and a linear component (Fig. 2). The linear decrease, which is a measure of the loss of 'remaining' cytoplasm and therefore probably of endogenous N metabolism, is greater in the male than in the female rat. On the other hand, the exponential decrease, which is a measure of 'labile' cytoplasm, proceeds considerably more slowly in the male than in the female rat. In other words, under identical dietary conditions the male rat forms more 'labile' liver cytoplasm and retains it better than the female.

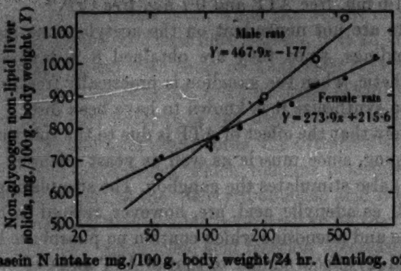

Fig. 1.

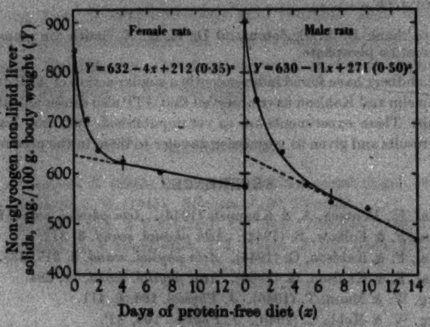

Fig. 2.

We are indebted to Dr D. N. Lawley for the statistical evaluation of the results and to the Medical Research Council for grants for expenses and scientific assistance (to H.W.K.).

Modified van Gieson stain. By A. Marshall.

Department of Physiology, University New Buildings, Edinburgh

The van Gieson stain for collagen fibres and muscle cytoplasm, although giving fairly good results, fades after a short period. The method described below overcomes this disadvantage and differentiates muscle cytoplasm, collagen and elastic tissue more clearly than van Gieson. It appears to be permanent as sections have not faded to any appreciable extent after a period of 2 years.

METHOD

(1) Haemalum, 15 min., followed by tap water.
(2) Aurantia, 0·5% solution in 70% alcohol, 5 min.
(3) Wash in distilled water, two or three changes.
(4) van Gieson's solution, 3 min.
(5) Wash in distilled water. Blot.
(6) Dehydrate in 95% and then in absolute alcohol; pass through rapidly.
(7) Clear in xylol and mount in *neutral* xylol dammar or balsam.

RESULTS

Nuclei, bluish black.
Muscle cytoplasm, brownish yellow.
Collagen, bright red.
Elastic laminae, bright yellow.
Red blood cells, yellow.

Although the muscle cytoplasm, elastic laminae and red cells all stain yellow, they are easily distinguishable by virtue of the differences in tint. The gleaming yellow of elastic fibres is particularly striking.

PROCEEDINGS
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28 *September* 1946

Potentiation of the vaginal response to oestrone by the 'relaxin' fraction of pregnant rabbit serum.

By A. D. Dewar, K. Hall and W. H. Newton. *Physiological Laboratory, Department of Physiology and Histology, The University of Liverpool*

Potentiation of oestrogen by relaxin is a possible common factor in the action of the latter on the mouse (Hall & Newton, 1946) and guinea-pig (Abramowitz, Money, Zarrow, Talmage, Kleinholz & Hisaw, 1944) symphysis pubis, and on the rat mammary gland (Hamolsky & Sparrow, 1945). Its action on the vaginal smear reaction is, therefore, being investigated.

Eighty oophorectomized mice were divided into six groups, two of which were given 0·25 μg., two 1 μg. and the other two 2 μg. of oestrone per mouse in a single dose in oily solution. One group on each dose received in addition a total of 0·25 c.c. per mouse of the relaxin fraction of pregnant rabbit serum (Abramowitz, Hisaw, Kleinholz, Money, Talmage & Zarrow, 1942). This was given in two doses; the first of 0·1 c.c. 24 hr. after the oestrone, and the second of 0·15 c.c. 6 hr. later. The experiment was then repeated with the groups reversed. The vaginal smears were followed and a 'score' of 4 was given for a series progressing to a full squamous cell smear (*S*), 1 for a full nucleated smear (*N*) with no further development, and 2 and 3 for nucleated smears progressing to different but incomplete degrees of cornification (e.g. *NS*, *NLS*, *LS*), the leucocyte (*L*) invasion occurring before the development of the full oestrous smear. The results were as follows (maximum score, 4 per mouse):

Treatment	No. of mice	Observed score	Percentage response
0·25 μg. oestrone	38	16·5	10·9
0·25 μg. oestrone + 'relaxin' extract	39	104·5	67·0
1 μg. oestrone	20	29·5	36·9
1 μg. oestrone + 'relaxin' extract	20	55·5	69·4
2 μg. oestrone	20	40·0	50·0
2 μg. oestrone + 'relaxin' extract	20	64·0	80·0

We conclude that the action of oestrone is potentiated by a substance in the relaxin extract of pregnant rabbit serum. The results remain the same by different methods of assessment, e.g. by allotting a score to each individual smear, or by observing duration of action. From a regression line constructed from the 'oestrone-alone' results, the 1 μg. and 2 μg. doses appear to be potentiated 75 times and the 0·25 μg. dose 50 times.

A similar extract of non-pregnant rabbit serum slightly potentiated the 0·25 μg. and the 1 μg. doses. Relaxin extract alone had no oestrogenic effect.

Our thanks are due to the Medical Research Council for a grant towards expenses and to Miss Maureen Young for a timely gift of pregnant rabbit serum.

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Observations on the central fovea of certain red-green-blind subjects.

By E. N. Willmer, *Physiology Laboratory, Cambridge*

König, in 1894, originally called attention to the blue-blindness of the central fovea of the normal human eye. This observation has recently been confirmed and the behaviour of the foveal area of the normal subject has been further investigated (Willmer, 1944; Willmer & Wright, 1945; Thomson & Wright, 1946).

Opportunity has now arisen to investigate the properties of this area in certain red-green-blind subjects. It has been found that, when vision is limited to the foveal centre, some of these subjects behave like monochromats and can match any colour with any other colour, provided that the brightness of the two matching fields is made identical. The two fields used in these experiments each subtended an angle of 10 min. at the eye, and they were separated by a space also subtending an angle of 10 min., in the centre of which was placed a minute white fixation point. The fields were illuminated by relatively pure spectral colours, obtained by the use of Ilford spectral filters. Similar results were also obtained when adjacent matching fields were used.

So far, three subjects have been found who have no difficulty in matching red with any other colour in the spectrum, including violet, so long as the brightness of the two fields is the same and the fields are centrally fixated. Three more can match red with any colour between red and blue, but can generally distinguish violet from red. The foveal centre of the first three is presumably monodic (i.e. it has only one pathway from the retina to the colour discrimination centre, assuming the colour-blindness to be peripheral in origin). An investigation of the spectral sensitivity of the foveal centre in such subjects is likely to provide information with regard to the nature of the pathway present and is now being made with the collaboration of Dr W. D. Wright and Dr L. C. Thomson.

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A method for direct microscopical study of living embryo bone fragments engrafted on the chick chorioallantoic membrane.

By N. M. Hancox, *Histological Laboratory, Department of Physiology and Histology, University of Liverpool*

Small fragments of embryo bone are attached to a coverslip by a plasma clot. The slip is inverted over a shell-hole exposure of the membrane which is then manœuvred into contact with the slip surface and the preparation incubated. The procedure is carried out aseptically. Incident illumination, or, for higher magnifications, the Leitz Ultropak, is employed to follow the development of the vascular pattern and subsequent osteogenesis, the specimen being kept warm in a simple type of stage incubator box.

Application of the London cannula technique to sheep and goats.

By R. A. Gregory, *Physiological Laboratory, University of Liverpool*

In a study of the absorption of carotene and other substances from the ruminant digestive tract (Goodwin, Dewar & Gregory, 1946), London cannulae were placed on the portal vein in sheep and goats by the following one-stage technique, which is based upon the two-stage procedure used for dogs by Crandall (1946).

Under closed-circuit cyclopropane-oxygen anaesthesia, the abdomen is opened by a right paracostal incision 10–12 in. long, and the portal vein exposed by retraction of the liver, pancreas and duodenum. The cannula is made from thin-walled silver tubing $\frac{1}{8}-\frac{3}{16}$ in. internal diameter, bearing at one end a perforated metal flange $\frac{1}{2}$ in. diameter, set at an angle of 45°. Along the tube a butterfly-shaped flange slides stiffly on a split cuff. The cannula is introduced into the abdominal cavity through a stab-wound made at a suitable place in the flank, so that the flange rests easily of its own accord on the portal vein. It is then secured there with four or more interrupted sutures. The butterfly flange is moved down the cannula as near to the vein as possible, and omentum wrapped around it. This flange subsequently becomes attached to the vein and adjacent structures by connective tissue, which maintains a firm union between cannula and vein after the original sutures have come away. The abdomen is closed in three layers with strong nylon sutures.

Blood may be taken from the portal vein 10 days or more after operation. The instrument used is a No. 20 B.W.G. hypodermic needle joined to a wider bore metal tube of suitable length. This in turn is connected by a flexible rubber junction to a syringe. The cannula is first washed out with warm boiled saline using a gum-elastic catheter attached to a syringe, and residual fluid removed by suction. The needle is now passed down the cannula until the slight resistance of the vein wall is encountered; it is then pressed onward gently for

$\frac{1}{4}$–$\frac{1}{2}$ in., applying suction with the syringe. Entry into the vein is indicated by a free flow of blood into the syringe.

I am indebted to Prof. L. A. Crandall, Jr., of the University of Tennessee, for generously communicating to me the details of his technique for the London cannula in dogs. I also wish to thank Mr J. McCarthy of this Department, who constructed the cannulae, for his valuable co-operation and suggestions.

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PROCEEDINGS
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Arterial spasm in the human umbilical cord. By A. F. Rogers (introduced by R. J. Brocklehurst), *Department of Physiology, University of Bristol*

As a part of a study of the behaviour of isolated arteries, fresh normal full term human umbilical cords were used as a source of material free from the complication of nerve supply. One artery in a 10 cm. length of umbilical cord was cannulated and the cord placed in a plethysmograph. This was placed in a constant temperature water-bath at 37° C. Ringer-Locke fluid (water 100, NaCl 0·9, KCl 0·042, $CaCl_2$ 0·024, $NaHCO_3$ 0·02) was supplied from a reservoir at such a height that the pressure at the artery was approximately that normally found in the cord. Oxygen containing 5 % carbon dioxide was bubbled through

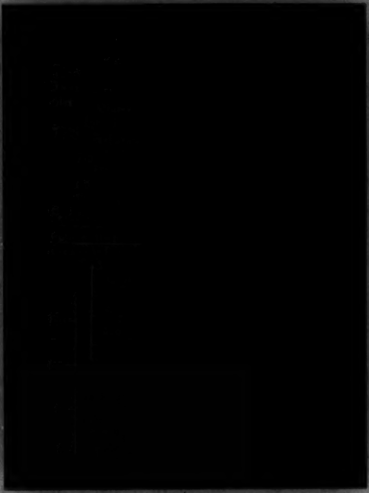

Fig. 1.

the reservoir, the gas escaping by a vent. *By closing and opening the vent the perfusion pressure could be suddenly or gradually raised and lowered again.* The perfusion fluid was heated and passed through a temperature stabilizing coil before entering the cannula. The outflow from the cord ran freely via a funnel into a closed vessel, displacing air which passed to a volume recorder. A motor, actuated by a time clock, opened this recorder to the atmosphere for one second in every thirty, permitting the recorder to return to zero.

Results. When the pressure was raised and allowed to fall to the previous pressure, provided the change was of sufficient duration and extent (Fig. 1), the artery went into spasm and perfusion ceased. The plethysmograph showed a decrease in cord volume, and the output recorder zero flow. The artery relaxed (usually fairly suddenly) and flow returned to normal, generally after a period of from ½ to 3 min.

The expense of this work was in part defrayed by a grant from the Colston Research Society.

The tolerance of sheep to low concentrations of blood sugar.

By M. W. S. Hitchcock and A. T. Phillipson, *The Agricultural Research Council's Unit of Animal Physiology, Cambridge*

The concentration of blood sugar in sheep is low compared to human standards and the range usually quoted is 40–60 mg.%. We have found, using Nelson's photometric adaptation of Somogyi's copper reduction method after cadmium precipitation (Nelson, 1944), that the concentration of sugar in samples of blood taken from non-pregnant ewes and wethers at different times of the day usually falls within the lower limits of this range and that, in apparently normal sheep, the concentration may be lower still. We have found values ranging from 21–61 mg.% in thirty-two samples of blood taken from seven sheep. Of these, 72% lay between 38 and 50 mg.%; 13% were higher and 16% were lower.

Table 1. The effect of insulin on the concentration of blood sugar

Experiment	1	2	3	4	5	6	7	8	9
Pre-dosing blood sugar (mg.%)	36	43	47	46	31	31	42	44	38
Units of insulin given:									
Dose 1	60	60	67	70	100	100	100	100	100
Dose 2	—	—	—	30	45	100	—	60	50
Dose 3	—	—	—	60	—	—	—	—	—
Duration of low blood sugar (hr.)	3	4½	1	6	4½	7	6	4	5½
Range of low blood sugar (mg.%)	5–17	8–17	13–17	10–18	9–21	10–20	10–19	8–20	13–17
Time, after dosing, of lowest blood sugar (hr.)	1¾	2½	2	1½*	2*	2½†	3	3¼†	2½*

* After first dose. † After second dose.

Injections of large doses of insulin reduce the blood sugar to the region of 10 mg.% or less, but no symptoms of insulin shock, other than a slight degree of lassitude, appear. The essential details of nine such experiments are given in Table 1. The method used for determining blood sugar in the first five experiments was that of King, Haslewood & Delory (1937).

The duration of the period of low blood sugar in Exps. 1, 4 and 6 may have exceeded the period stated, as sampling ceased before any significant rise occurred. In Exp. 6, the sheep was starved and the rumen emptied before dosing, so that there was no question of absorption of lower fatty acids from the rumen; but this animal showed no nervous symptoms.

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Effect of flavine-adenine-dinucleotide and other substances on the synthesis of acetylcholine.

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Saline extracts of acetone-dried brain and human placenta form large amounts of acetylcholine in the presence of adenosinetriphosphate (ATP), choline, KCl, Mg^{++}, citrate and a dialysable, heat-stable substance, the 'activator', prepared by boiling saline extracts of acetone-dried brain. In brain extracts, citrate as well as activator causes a large increase in the formation of acetylcholine, citrate being more effective. In placental extracts citrate has relatively less accelerating effect than activator (for references see Comline, 1946). It has now been found that flavine-adenine-dinucleotide (FAD) can replace the activator to a great extent in the synthesis of acetylcholine.

(1) Placental extracts synthesized relatively small amounts of acetylcholine when incubated for 1 hr. at 37° C. in the presence of cysteine, choline, KCl, Mg^{++}, ATP and citrate. However, if either activator or FAD was included in the sample the amount of acetylcholine formed per g. acetone-dried tissue was between 280 and 960 (average 690) μg. with activator, and between 120 and 740 (average 490) μg. with FAD. The concentration of FAD in the samples was between $0{\cdot}5$ and $0{\cdot}9 \times 10^{-5}$ M., which gave a maximal response.

(2) Experiments with dialysed placental extracts have shown that the strong activation by FAD is dependent on the presence of both Mg ions and citrate.

(3) FAD also accelerates the synthesis of acetylcholine by saline extracts of acetone-dried brain, but usually less than activator. When cysteine was omitted from the samples, however, FAD decreased the formation of acetylcholine; with placental extracts the absence of cysteine reduced but did not reverse the effect of FAD.

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Coenzyme I *and* II, added either together or separately, did not accelerate the synthesis of acetylcholine by placental or brain extracts. Reduced coenzyme I was also without effect.

Acetylphosphate and phosphorylcholine have been suggested (Nachmansohn & Machado, 1943) as possible intermediates in the formation of acetylcholine. Neither of these substances had any effect on the formation of acetylcholine by placental or brain extracts.

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